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Genome size variation among natural populations of *Brachypodium distachyon* and *B. hybridum* collected from different regions of Turkey

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Abstract: *Brachypodium distachyon* (L.) P.Beauv. is a model grass species that is useful for studying temperate cereal, forage, and energy crops. In this study we aimed to determine the genome size and species identity in the collections of the *B. distachyon* complex that included samples from 56 different locations in Turkey, applying chromosome counting and nuclear genome size evaluation by flow cytometry method (FCM). Moreover, the study examined whether diverse geographical conditions at the collecting sites influence the genome size. Chromosomal analysis revealed that the number of *B. distachyon* (x = 5, 2n = 10) genotypes was 48 and the number of *B. hybridum* (x = 5 + 10, 2n = 30) ones was 11. No genotypes of *B. stacei* were found. FCM analyses revealed that the minimum and maximum genome size of *B. distachyon* was 0.732 and 0.752 pg2C^{-1}, respectively. The mean genome size of *B. distachyon* was 0.743 pg2C^{-1}. The mean genome size of *B. hybridum* was 1.431 pg2C^{-1} with the minimum 1.417 pg2C^{-1} and maximum 1.451 pg2C^{-1}. Intraspecific variation in the genome size was identified for both species. It was determined that geographical origin (localization, altitude) had a statistically significant effect on genome size in *B. distachyon*. In addition, a negative correlation was found between altitude and genome size in this species. On the other hand, localization and altitude did not have any statistically significant effect on genome size in *B. hybridum*.

Key words: *Brachypodium distachyon*, *B. hybridum*, flow cytometry, genome size variation, model grass genus

1. Introduction

Brachypodium distachyon is a useful model organism to study various aspects of plant and grass biology (Vogel et al., 2010; Catalán et al., 2014; Scholthof et al., 2018). Due to its small and compact nuclear genome, diverse ecological tolerances, easy propagation under controlled growth conditions, and already existing considerable molecular and genomic resources, this plant is an excellent candidate in terms of addressing fundamental questions in comparative genomics and ecological studies. Furthermore, it is also advantageous in terms of conversion to cereal and biofuel crops (Catalán et al., 2014; Lopez-Alvarez et al., 2017).

In the first karyological analyses of *B. distachyon*, three different chromosomal numbers (2n = 10, 20, and 30) were identified and it was concluded that chromosomal races with 2n = 20 and 2n = 30 were autotetra- and autohexapolyploids, respectively (Robertson, 1981). Later, in their extensive phylogenetic, cytogenetic, and phenotypic analyses, Catalán et al. (2012) demonstrated that these three cytotypes should, in fact, be considered three different annual species (i.e. two diploids), each with a different chromosome base number: *B. distachyon* (x = 5, 2n = 10), *B. stacei* (x = 10, 2n = 20), and their derived allotetraploid *B. hybridum* (x = 5 + 10, 2n = 30). Through complex cytomolecular analyses using fluorescence in situ hybridization (FISH) with various probes, such as rDNA, total genomic DNA, and single-locus bacterial artificial chromosome (BAC)-based probes, some studies clearly showed that the genomes existing in the two diploid species participated in the origin of *B. hybridum* (Hasterok et al., 2004, 2006a, 2006b; Catalán et al., 2012; López-Alvarez et al., 2012; Scholthof et al., 2018). Moreover, despite having two times higher chromosome numbers, the genome size of *B. stacei* (0.564 pg2C^{-1}) is roughly similar to that of *B. distachyon* (0.631 pg2C^{-1}); however, the genome size of *B. hybridum* corresponds to the sum of the two progenitor genomes (1.265 pg2C^{-1}) (Catalán et al., 2012; Scholthof et al., 2018).

It is known that these three species are native to the entire circum-Mediterranean region (Garvin et al., 2008; Catalán et al., 2012). They can grow in different environments and at different latitudes and altitudes,

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which represent a wide range of biotic and abiotic conditions that may be associated with adaptive natural genetic variation (Garvin et al., 2008; Manzaneda et al., 2012). According to the studies using environmental niche modeling analysis, while *B. distachyon* generally grows in higher, cooler, and wetter places north of N30°, *B. stacei* grows in lower, warmer, and drier places south of N40°30′. Moreover, *B. hybridum* grows in places with intermediate ecological features and across the latitudinal boundaries of its two diploid ancestors. On the other hand, it is more often observed that *B. distachyon* overlaps with *B. stacei* (López-Alvarez et al., 2015; Catalán et al., 2016b).

Furthermore, statistical analyses of morphometric traits showed that when these three species were grown under controlled greenhouse conditions eight characters, namely leaf stomatal guard cell length, pollen grain length, plant height, culm leaf-blade width, panicle length, number of spikelets per panicle, lemma length, and awn length, significantly differentiated among them (Catalán et al., 2016b; López-Alvarez et al., 2017). Although the three species can be differentiated with respect to their several phenotypic and cytogenetic traits (Catalán et al., 2012; López-Álvarez et al., 2012; Betekhtin et al., 2014; Lusinska et al., 2018), identification of them based on morphological features is not always straightforward since their wild populations show overlapping phenotypic variation for some characters (Catalán et al., 2016b). Therefore, when using currently employed identification methods such as morphology, this situation has caused uncertainty in the taxonomy of the model species and its close allies or even caused misclassifications of the model (López-Alvarez et al., 2012; Catalán et al., 2016a). Recent studies have addressed the impact of environmental factors, such as drought in particular (Manzaneda et al., 2012; Bareither et al., 2017) and ecological niches (López-Alvarez et al., 2015), on the distribution of these species.

Brachypodium germplasm collections have been assembled for wide variation and economically important traits (Filiz et al., 2009). Currently, one of the largest available collections is the Turkish collection established by Vogel et al. (2009), which comprises 187 diploid genotypes from 53 locations and 84 inbred lines (Budak et al., 2014). Our collection is also a part of this collection. Some of the genotypes from our collection have been used as biological materials in various research related to genetic diversity (Filiz et al., 2009; Vogel et al., 2009), cytogenetic analyses (Wolny and Hasterok, 2009), genome structure (Dinh Thi et al., 2014), diversity (Gordon et al., 2014), molecular phylogeny analyses (López-Alvarez et al., 2012), ecological niches (López-Alvarez et al., 2015), drought tolerance (Tatlı et al., 2015; Luo et al., 2016), and pan-genome correlations (Gordon et al., 2017). Meanwhile, new materials have been added to our collection. Therefore, better and more up-to-date characterization is required in order to determine which of the *Brachypodium* annuals are behind the seed populations in our collection. The purposes of this study are: (i) to identify chromosome numbers and genome sizes of the populations in the *Brachypodium* collection from various regions of Turkey with different ecological and geographical conditions; (ii) to determine the taxonomic identity of the samples and natural distribution areas for each sample within the collection; and (iii) to examine the effects of geographical origin (localization, altitude) on genome size.

2. Materials and methods

2.1. Plant material

Populations of *Brachypodium* from 56 geographical areas of Turkey were analyzed. They have been included in the Genetic Resources Collection of the Department of Field Crops and are naturally present in the flora of the Marmara, Aegean, Mediterranean, Central Anatolia, and Southeastern Anatolia regions of the country. Code numbers and attributes of the geographical collection areas of the populations are shown in Table 1.

2.2. Germination of seeds

Seeds were sown in multiple plastic pots containing a turf/perlite mixture. Sowing was conducted as three replicates. After 2 months, the plantlets were transferred to larger pots (7 × 7 cm) also containing a turf/perlite mixture. The plants were grown in a plastic greenhouse and they were monitored on a daily basis.

2.3. Chromosome analysis

In the first phase of the study, a chromosome count was performed in order to determine the identity of *Brachypodium* species. Five plants per population were analyzed. Root tips (1–1.5 cm) were harvested from adult plants growing in the greenhouse and treated with cold water for about 20 h before fixation in ethanol:glacial acetic acid (3:1, v/v). The chromosome preparations were stained using the Feulgen reaction. More specifically, fixed material was hydrolyzed for 3.5–4 min using 1 N HCl at 60 °C. Then the material was transferred into Schiff's reagent at room temperature. Root tips of *B. distachyon* were squashed in acetocarmine and examined using a bright-field microscope (Olympus BX51) (Figure 1). However, the chromosomes of *B. hybridum* in Figure 2 became apparent after DAPI, which is visualized by epifluorescence microscopy, not by the bright-field microscopy. Images of full chromosome complements were taken using a CCD digital camera (Spot RT Slider) attached to the microscope.

2.4. Determination of the genome size

Genome size was determined using the flow cytometry method (FCM). Suspensions of intact nuclei were prepared using commercial kits (Sysmex). Rice (*Oryza*

Table 1. Population code numbers, location, latitude, longitude, altitude, chromosome numbers, mean genome size, standard deviation, and significance group of *B. distachyon* and *B. hybridum* populations from different geographical regions of Turkey in this study.

Population code numbers	Location	Latitude	Longitude	Altitude (m)	Chromosome numbers (2n)	Mean of genome size (pg $2C^{-1}$), ±SD	Significance group
Bd13	Büyük Karıştıran - Kırklareli	N41°17′39.2″	E027°32′82.4″	8	10	0.752 ± 0.009	a
Bd105	Kütahya Tavşanlı exit	N39°32′22.2″	E029°38′01.4″	1011	10	0.752 ± 0.006	a
Bd81	Çanakkale Şehitlik	N40°15′33.9″	E026°18′77.4″	261	10	0.751 ± 0.007	ab
Bd37	Barağı - Keşan - Edirne	N40°43′18.7″	E026°25′90.6″	18	10	0.75 ± 0.006	abc
Bd93	Balya -Yenice II	N39°46′88.8″	E027°24′37.5″	522	10	0.75 ± 0.013	abc
Bd94	Balıkesir Center	N39°38′74.1″	E027°46′10.0″	248	10	0.75 ± 0.011	abc
Bd8	Bıyıkali - Tekirdağ (2)	N41°02′02.8″	E027°22′16.2″	145	10	0.749 ± 0.008	abc
Bd4	Çiftlik - Uzunköprü - Edirne	N41°15′68.6″	E026°37′29.8″	33	10	0.748 ± 0.013	a–d
Bd66	Şehitlik II Çanakkale	N40°14′65.3″	E026°17′70.8″	182	10	0.748 ± 0.005	a–d
Bd62	Hasköy - Enez - Edirne	N40°38′71.7″	E026°16′34.2″	37	10	0.748 ± 0.011	a–d
Bd91	Beginning of Çanakkale Bursa Road	N38°43′86.4″	E034°49′91.0″	47	10	0.747 ± 0.010	a–e
Bd42	Hasköy - Enez - Edirne	N40°38′71.7″	E026°16′34.2″	37	10	0.747 ± 0.006	a–e
Bd3	Çakmak - Edirne	N41°23′43.8″	E026°39′63.7″	85	10	0.747 ± 0.006	a–e
Bd96	Pada Village Kepsüt - Dursunbey, Balıkesir	N39°44′66.9″	E028°21′42.3″	498	10	0.747 ± 0.010	a–e
Bd14	Kayı Village - Tekirdağ	N41°02′53.7″	E027°30′37.4″	216	10	0.747 ± 0.005	a–e
Bd69	Ilgardere - Gelibolu - Çanakkale	N40°15′88.9″	E026°28′85.9″	18	10	0.747 ± 0.010	a–e
Bd84	Ağva Şile road II - Istanbul	N41°05′34.7″	E029°45′24.9″	132	10	0.746 ± 0.010	a–f
Bd90	18 Mart Univ. Campus - Çanakkale	N40°06′91.0″	E026°25′48.2″	125	10	0.745 ± 0.008	a–g
Bd95	40 km to Balıkesir past Balya	N39°42′07.3″	E027°33′28.9″	357	10	0.745 ± 0.009	a–g
Bd63	Yenice - Balya	N39°48′00.6″	E027°22′94.8″	350	10	0.745 ± 0.007	a–g
Bd83	TÜBİTAK - MAM, İzmit	N40°47′18.9″	E029°27′60.1″	178	10	0.744 ± 0.010	a–h
Bd38	Kılıçköy - Keşan - Edirne	N40°47′76.8″	E026°34′28.2″	39	10	0.744 ± 0.009	a–h
Bd65	Balıkesir center II Kütahya exit	N39°41′13.1″	E027°58′78.2″	193	10	0.744 ± 0.013	a–h
Bd12	Buzağıcı - Hayrabolu - Tekirdağ	N41°15′349″	E027°08′40.0″	53	10	0.744 ± 0.007	a–h
Bd41	İzzetiye - Keşan Edirne	N40°48′349″	E026°39′61.5″	99	10	0.744 ± 0.008	a–h
Bd97	Kütahya road Dursunbey - Harmancık	N39°39′30.6″	E029°01′93.3″	606	10	0.744 ± 0.014	a–h
Bd11	Çeneköy, Hayrabolu	N41°12′02.9″	E027°11′19.2″	83	10	0.743 ± 0.007	a–i
Bd68	Ilgardere - Gelibolu - Çanakkale	N40°15′88.9″	E026°28′85.9″	18	10	0.743 ± 0.009	a–i
Bd99	Muğla	N37°07′54.5″	E028°22′72.4″	624	10	0.743 ± 0.005	a–i
Bd117	10 km from Kütahya	N39°30′12.6″	E029°52′61.8″	1035	10	0.743 ± 0.006	a–i
Bd15	Yeşilsırt Village - Muratlı - Tekirdağ	N41°11′70.4″	E027°47′33.4″	180	10	0.742 ± 0.006	b–i
Bd112	Kaymaz exit of Eskişehir	N39°29′65.0″	E031°14′47.3″	1017	10	0.741 ± 0.007	b–k
Koz	Kozluk - Batman	N38°9′82.6″	E41°36′34.8″	853	10	0.741 ± 0.045	b–k
Bd67	Harmancık - Kütahya	N39°40′77.3″	E029°08′84.6″	672	10	0.74 ± 0.008	c–k
Gaz	Gaziantep	N37°7′39.8″	E37°23′26.9″	891	10	0.739 ± 0.008	d–k
Bd108	Alanya - Antalya	N36°36′53.9″	E031°48′33.3″	18	10	0.739 ± 0.007	d–k
Bd100	Kütahya - Eskişehir	N39°34′64.3″	E030°07′20.8″	927	10	0.738 ± 0.009	e–k
Bd88	Avanos III Nevşehir	N38°44′53.6″	E034°50′28.9″	1139	10	0.738 ± 0.009	e–k
Bd16	Seymen - Çorlu - Tekirdağ	N41°05′78.6″	E027°5′79.6″	107	10	0.738 ± 0.008	e–k

Table 1. (Continued).

Adi	Adıyaman	N37°46′14.5″	E38°21′ 8.2″	510	10	0.738 ± 0.003	e–k
Bd92	Dursunbey - Balıkesir	N39°36′51.5″	E028°37′93.1″	665	10	0.737 ± 0.011	f–k
Bd85	35 km from Nevşehir	N38°50′44.0″	E034°33′26.6″	1174	10	0.736 ± 0.006	g–k
Bd86	Avanos - Nevşehir	N38°44′47.0″	E034°50′72.5″	1092	10	0.735 ± 0.009	hik
Bd89	Avanos - Nevşehir	N38°43′86.4″	E034°49′91.0″	967	10	0.735 ± 0.007	hik
Kah	Kahta - Adıyaman	N37°44′2.3″	E38°32′0.2″	665	10	0.735 ± 0.077	hik
Bd111	Polatlı - Haymana	N39°29′64.5″	E032°26′81.0″	974	10	0.735 ± 0.010	hik
Bd109	Polatlı - Haymana, Polatlı exit	N39°32′59.7″	E032°13′90.9″	980	10	0.734 ± 0.010	ik
Bis	Bismil - Diyarbakır	N37°52′35.6″	E41°0′54.3″	529	10	0.732 ± 0.004	k
Average genome sizes					0.743 ± 0.005	MSE = 0.00081	
Bhyb114	Köyceğiz exit of Muğla	N36°59′52.0″	E028°39′29.1″	33	30	1.451 ± 0.038	a
Bhyb77	Bozyazı - Mersin	N36°05′81.5″	E032°56′12.8″	17	30	1.442 ± 0.010	ab
Bhyb73	Milas - Muğla	N37°21′67.4″	E027°41′21.7″	42	30	1.436 ± 0.023	abc
Bhyb103	Anamur - Gazipaşa, Meleç Village Mersin	N36°02′46.4″	E032°42′06.6″	136	30	1.435 ± 0.011	a–d
Bhyb106	Anamur - Gazipaşa, Meleç Village Mersin	N36°02′46.4″	E032°42′06.6″	136	30	1.432 ± 0.012	bcd
Bhyb113	Ölüdeniz - Fethiye	N36°33′01.3″	E029°07′54.2″	20	30	1.429 ± 0.012	bcd
Bhyb107	Alanya - Antalya	N36°36′53.9″	E031°48′33.3″	18	30	1.427 ± 0.022	bcd
Bhyb122	Kuşadası -Aydın	N37°47′95.5″	E027°18′27.9″	134	30	1.424 ± 0.009	bcd
Bhyb102	Taşucu - Aydıncık, Mersin	N36°15′69.4″	E033°48′22.4″	211	30	1.423 ± 0.011	cd
Bhyb110	Beginning of Antalya - Korkuteli road	N36°57′50.6″	E030°35′57.0″	300	30	1.421 ± 0.012	cd
Bhyb124	Aydıncık exit of Mersin	N36°07′92.5″	E033°16′70.2″	69	30	1.417 ± 0.016	d
Average genome sizes					1.431 ± 0.010	MSE = 0.00033	

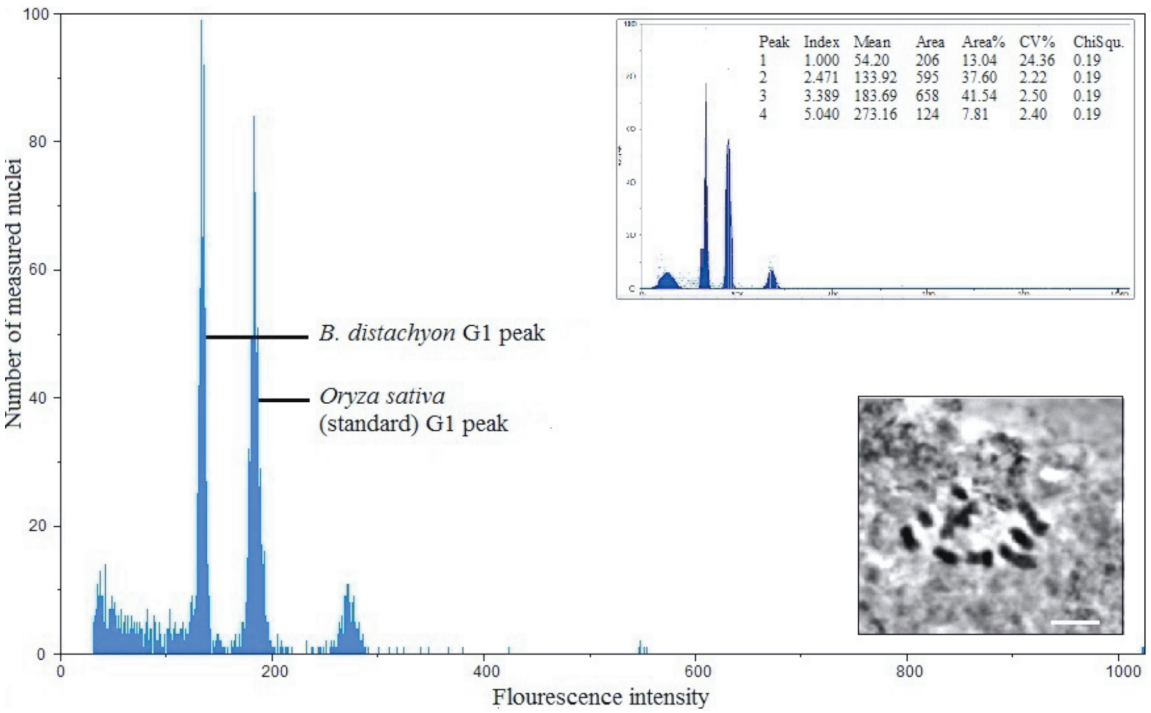

Figure 1. Relative G1 peak positions of *B. distachyon* (Bis-5) and the standard (*Oryza sativa*). Photomicrograph shows mitotic metaphase chromosomes of Bis-5. Scale bar: 5 µm.

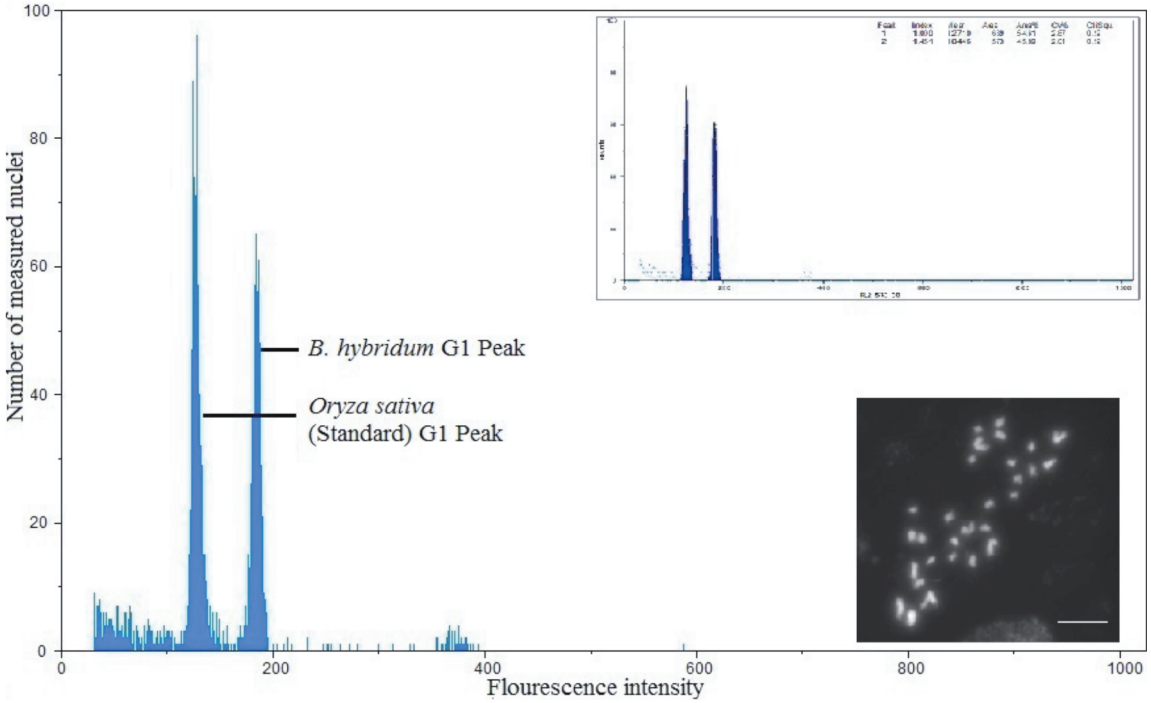

Figure 2. Relative G1 peak positions of *B. hybridum* (Bhyb 77) and the standard *Oryza sativa*). Photomicrograph shows mitotic metaphase chromosomes of Bhyb 77. Scale bar: 5 µm.

sativa; 0.99 pg2C^{-1} DNA) was used as a standard. A sample of the fresh leaf with an area of approximately 0.5 cm^2 and the fresh leaf of the internal standard were simultaneously chopped in a petri dish containing 0.5 mL of extraction buffer. The homogenized solution was transferred into a glass tube through a 30-µm filter and then 2 mL of staining buffer (CyStain PI Absolute P) was added to each tube. The samples were incubated at room temperature in the dark for at least 1 h before the FCM analysis. Ten plants per population were analyzed individually and 5000 nuclei were analyzed in each sample using a CyFlow Space cytometer (Sysmex). The genome size of the *Brachypodium* samples was calculated based on relative positions of the G1 peaks of the sample and the standard.

2.5. Statistical analysis

Variance analysis and Duncan's test were performed using the general linear model in Statistical Analysis Software (SAS). Through this approach, the statistical significance of differences between genome sizes of populations and the effect of geographical areas and geographical regions on genome size of populations were analyzed. The effect of geographical distance on genome size was determined using the Mantel test and correlation analysis was performed in order to determine the relationship between altitude and genome size. The effect of altitudinal changes on genome size was examined using regression analysis.

3. Results and discussion

According to the chromosomal analysis, while *B. distachyon* (x = 5, 2n = 10) (Figure 1) was identified in 48 of the studied populations, *B. hybridum* (x = 5 + 10, 2n = 30) was identified in the remaining 11 populations (Figure 2). However, *B. stacei* was not found. The obtained results are consistent with previous findings. For example, López-Alvarez et al. (2012, 2015) also identified the presence of only *B. distachyon* and *B. hybridum* populations in Turkey.

The FCM was used to determine genome sizes in the samples studied. We obtained good quality G1 peaks with CVs lower than 3%, indicating precise measurements (Figures 1 and 2). The minimum and maximum genome sizes for *B. distachyon* were 0.732 and 0.752 pg2C^{-1}, respectively, and the mean genome size determined for this species was 0.743 pg2C^{-1} (Table 1). Our results are similar to those in previous studies (Shi et al., 1993; Draper et al., 2001; Bennett and Leitch, 2005; Filiz et al., 2009; Wolny and Hasterok, 2009; Catalán et al., 2012; Dinh Thi et al., 2016; Scholthof et al., 2018) and the minor discrepancies can be due to either the intraspecific variation in the genome size of *B. distachyon* or the use of a different internal standard (Dolezel and Bartos, 2005).

The mean genome size of *B. hybridum* was 1.431 pg2C^{-1} with the minimum and maximum values of 1.417 pg2C^{-1} and 1.451 pg2C^{-1}, respectively. These values are consistent with the previously published data (Catalán et al., 2012;

Dinh Thi et al., 2016; Scholthof et al., 2018), and, as in the case of *B. distachyon*, some slight differences can be attributed to some methodological peculiarities (Dolezel and Bartos, 2005).

Moreover, among the *B. distachyon* populations, while the Bis population had the lowest nuclear DNA content (0.732 pg2C^{-1}), Bd13 had the highest (0.752 pg2C^{-1}). In the case of *B. hybridum*, while Bhyb124 had the smallest nuclear genome size (1.417 pg2C^{-1}), Bhyb114 had the largest (1.451 pg2C^{-1}) among all populations examined in the present study. In our analyses, we detected interpopulation variability in the genome size of *B. distachyon* and *B. hybridum* in Turkey. This was probably due to the fact that different populations grow in different environments and at different latitudes and altitudes in a wide range of biotic and abiotic conditions that may shape natural genetic variation (Garvin et al., 2008; Manzaneda et al., 2012; López-Alvarez et al., 2015; Catalán et al., 2016b).

The genome size variations observed were statistically significant ($P < 0.01$) in both species and the populations formed different groups in Duncan's test (Table 1). The reasons for intraspecific variation have been examined in many studies. For example, the ones that are likely to cause such a variation are, inter alia, the amount of repetitive DNA in the genome that can be different in different populations due to the accumulation of mobile elements (Muñoz Diez et al., 2012; Tenaillon et al., 2016), chromosome size (Gregory, 2005), differences in cytosolic structure and changes in levels of secondary metabolites in different seasons (Noirot et al., 2005), sometimes also innate methodological limitations (Draper et al., 2001), location (Kalendar et al., 2000), ecological and geographic changes (Knight et al., 2005), genome mutation (Lynch et al., 2011), and positive natural selection (Knight and Beaulieu, 2008). In addition, differences in climate (especially precipitation) and geography (region, altitude, latitude) have been associated with different genome sizes and ploidy levels (Manzaneda et al., 2012; López-Alvarez et al., 2015; Bareither et al., 2017). Recent phylogenetic studies have suggested that there is a link between genome size and the gene content of the plant. Gordon et al. (2017) examined the genetic structure of *B. distachyon* and found three groups of populations, which were distinguished as EDF+ (extremely delayed flowering), T+ (Turkey and other countries), and S+ (Spain and other countries). EDF+ and T+ groups have their natural distribution in Turkey. The flowering time of populations and geographical isolation apparently affect the speciation of these groups (Ream et al., 2014; Gordon et al., 2017). This study by Gordon et al. revealed that the EDF+ population had distinct polymorphisms in the vernalization and flowering genes. It was also shown that the three populations differed greatly in their pan-genome composition, i.e. while hundreds of pan-genes are core to one subpopulation, they are absent in other populations (Gordon et al., 2017).

In our study, the EDF+ samples with 0.745 pg2C^{-1} (minimum: 0.735 pg2C^{-1}, maximum: 0.752 pg2C^{-1}) mean genome sizes and the T+ samples with 0.740 pg2C^{-1} (minimum: 0.732 pg2C^{-1}, maximum: 0.751 pg2C^{-1}) genome sizes (Table 2) also showed that the association to one or more genetic groups might have caused variations in genome size. The genome size of the EDF+ group was higher than that of the T+ group. Additionally the genome size variations were statistically significant ($P < 0.01$) for both groups. In the present study, the flowering time was measured in days without applying vernalization and photoperiod for all populations. The flowering time of the populations ranged from 88 to 149 days (mean: 114 days) and the difference was statistically significant ($P < 0.01$).

As shown in Table 1, while samples collected from the same or nearby areas ended up in different groups, samples collected from distant areas were grouped in the same one. It was revealed that the genome sizes of the populations were affected by their geographical origin. However, this effect was not statistically significant. Our results are consistent with those of similar studies that infer ecological and climatic factors affected genome size and ploidy levels (Knight et al., 2005; Manzaneda et al., 2012; López-Alvarez et al., 2015; Bareither et al., 2017).

The effects of geographical distances on the genome size of plants were also analyzed using the Mantel test. The Rxy value (correlation coefficient of the Mantel test) was calculated as 0.415 ($P < 0.01$) for *B. distachyon* (Figure 3A). The fact that *B. distachyon* has a self-fertilization feature (Vogel et al., 2009) affects the distribution distances of the pollen and seeds, and thereby it may also cause the geographical distances to have a significant effect on genome size. Moreover, as the distance within the geographical area increases, the climatic and ecological characteristics of the region might change. Therefore, as depending on the geographical distance, it is clear that these features may influence the genome size as emphasized in previous studies (Knight et al., 2005; López-Alvarez et al., 2015; Bareither et al., 2017).

On the other hand, the *B. hybridum* Rxy (–0.120) value was within the random permutations (Figure 3B) and the P-value was calculated as 0.327. Therefore, the relationship between geographical distances and genome sizes was not statistically significant for *B. hybridum*. This result was interesting since the samples were sourced from ten different locations close to or away from each other. Only the fact that *B. hybridum* has a natural distribution in Turkey and it spreads especially in the coastal regions of the Aegean and Mediterranean regions where the climatic and geographic factors are similar supports this result.

Table 2. Population code numbers, flowering time, genetic structure group, and mean genome size of *B. distachyon* populations from different geographical regions of Turkey in this study.

Population code numbers	Flowering time (days)	Genetic structure group	Mean of genome size (pg $2C^{-1}$), ±SD
Gaz.	88	T+	0.739 ± 0.008
Kah.	88	T+	0.735 ± 0.077
Adi	91	T+	0.738 ± 0.003
Bd99	93	T+	0.743 ± 0.005
Koz.	93	T+	0.741 ± 0.045
Bis.	95	T+	0.732 ± 0.004
Bd81	97	T+	0.751 ± 0.007
Bd83	97	T+	0.744 ± 0.010
Bd84	97	T+	0.746 ± 0.010
Bd86	97	T+	0.735 ± 0.009
Bd92	99	T+	0.737 ± 0.011
Bd90	101	T+	0.745 ± 0.008
Bd88	105	T+	0.738 ± 0.009
Bd62	106	T+	0.748 ± 0.011
Bd65	107	T+	0.744 ± 0.013
Bd93	108	T+	0.75 ± 0.013
Bd112	109	T+	0.741 ± 0.007
Bd69	110	T+	0.747 ± 0.010
Bd97	110	T+	0.744 ± 0.014
Bd67	111	T+	0.74 ± 0.008
Bd94	112	T+	0.75 ± 0.011
Bd111	112	T+	0.735 ± 0.010
Bd117	112	T+	0.743 ± 0.006
Bd91	113	T+	0.747 ± 0.010
Bd108	113	T+	0.739 ± 0.007
Bd100	114	T+	0.738 ± 0.009
Bd109	114	T+	0.734 ± 0.010
Bd96	118	T+	0.747 ± 0.010
Bd63	119	T+	0.745 ± 0.007
Average genome sizes		0.740 ± 0.008 pg$2C^{-1}$	MSE = 0.003
Bd41	120	EDF+	0.744 ± 0.008
Bd85	121	EDF+	0.736 ± 0.006
Bd89	121	EDF+	0.735 ± 0.007
Bd95	121	EDF+	0.745 ± 0.009
Bd37	125	EDF+	0.75 ± 0.006
Bd105	128	EDF+	0.752 ± 0.006
Bd4	129	EDF+	0.748 ± 0.013
Bd13	129	EDF+	0.752 ± 0.009
Bd66	129	EDF+	0.748 ± 0.005
Bd3	130	EDF+	0.747 ± 0.006

Table 2. (Continued).

Bd12	130	EDF+	0.744 ± 0.007
Bd14	130	EDF+	0.747 ± 0.005
Bd38	130	EDF+	0.744 ± 0.009
Bd8	131	EDF+	0.749 ± 0.008
Bd16	131	EDF+	0.738 ± 0.008
Bd42	133	EDF+	0.747 ± 0.006
Bd68	134	EDF+	0.743 ± 0.009
Bd15	136	EDF+	0.742 ± 0.006
Bd11	149	EDF+	0.743 ± 0.007
Average genome size		0.745 ± 0.007	MSE = 0.0001
EDF+ (extremely delayed flowering), T+ (Turkey and other countries)			

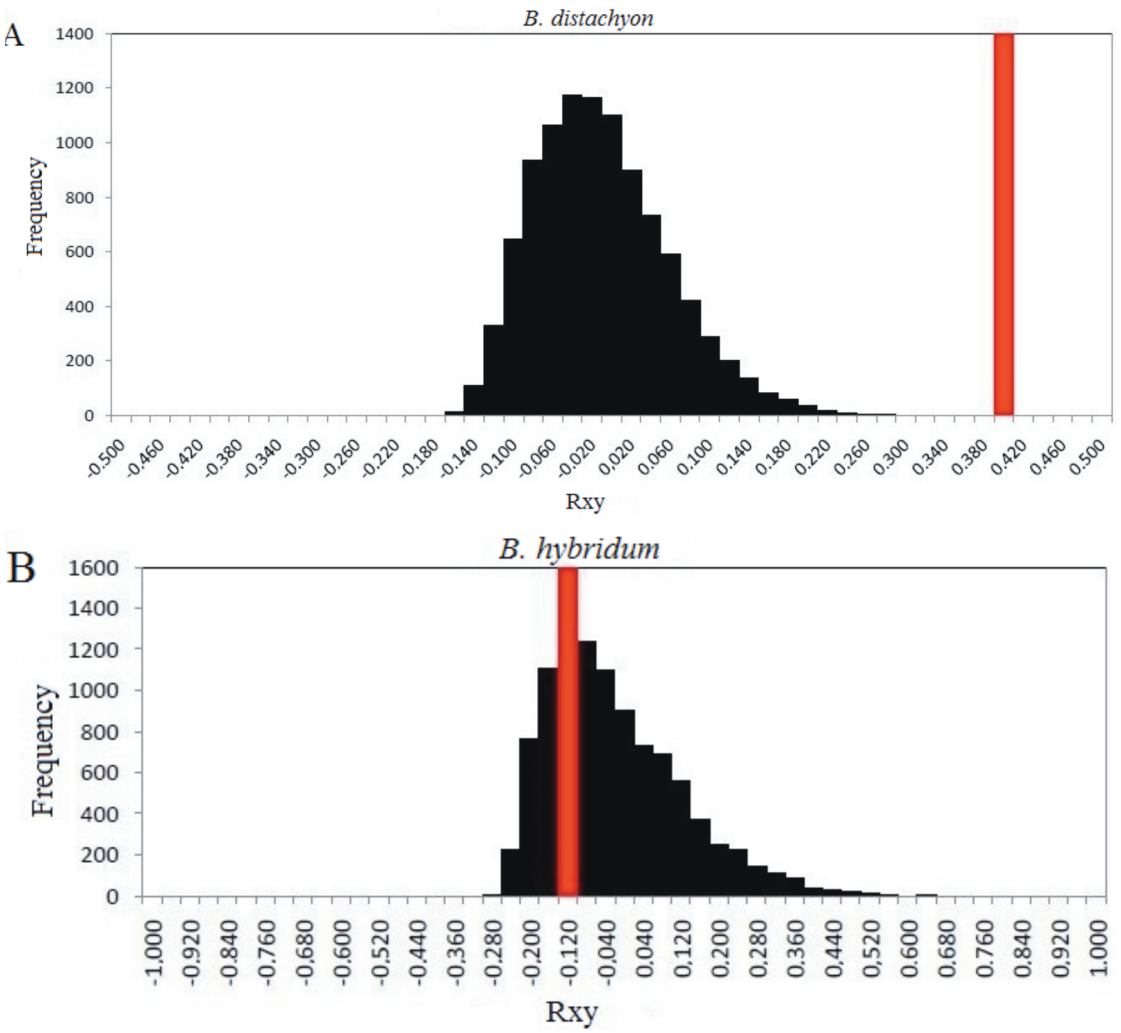

Figure 3. The results of the Mantel test determining the effect of geographical distance on genome size based on A: *B. distachyon* and B: *B. hybridum*. The figure shows the frequency distribution of random Rxy vs. observed Rxy. The Rxy value is correlated with the Mantel test coefficient.

The effect of geographical region on the sampling of intraspecific genome size variations was statistically significant for *B. distachyon* (P < 0.01). *B. distachyon* populations collected from the Marmara and Aegean regions were in the same group, while *B. distachyon* populations from the Central Anatolia and Southeastern Anatolia regions were in another group (Table 3). In addition, the data revealed that differences between genome sizes of *B. distachyon* populations growing in ecologically similar areas of Turkey were not statistically significant.

So far, many studies have analyzed the relationship between geographic region and genome size. Some of these studies reported that geographical region had an effect on interspecific genome size variation (Bennett, 1987; Kalendar et al., 2000; Smarda and Bures, 2006; Özkan et al., 2010). In a similar way to our results, Kisha et al. (2009) suggested that populations collected from different regions had different genetic characters, but that the genetic variation between populations caused by self-pollination was limited. Vogel et al. (2009) and Mur et al. (2011) noted that while there were correlations between genotypic variations no correlation was found in terms of the region and there was a high level of genotypic variation at different locations within regions. Manzaneda et al. (2012) highlighted that *B. distachyon* and *B. hybridum* had a region-based distribution on the Iberian Peninsula. Bareither et al. (2017) reported the same findings for the species of the *B. distachyon* complex in Israel. However, in our study, no statistically significant difference was found between the genome sizes of *B. hybridum* populations collected from the Aegean and Mediterranean regions (Table 2). This result can be explained by the relationship between geographical distance and genome size. In other words, even if the geographical region changes, the high ecological tolerance of *B. hybridum* might not affect its genome size (Catalán et al., 2016b; Scholthof et al., 2018).

In the present study, the effect of altitude on genome size was investigated using correlation analysis. It revealed that there was a negative correlation (–0.478**) between altitude and nuclear DNA content in *B. distachyon* (P = 0.0008). Regression analysis was used to determine how the increase in altitude affected genome size. The regression equation was y = –2E – 06X + 0.745 (Figure 4A). Based on this, it can be said that a one-unit increase in altitude (x) caused a statistically significant decrease in nuclear DNA content in *B. distachyon* (P < 0.01). By contrast, there was no significant change in the genome size of *B. hybridum* (P = 0.122) (Figure 4B). As in the case of the *B. hybridum* genotypes used in this study, studies on *Picea glauca* (Teoh and Rees, 1976) and *Vicia faba* (Ceccarelli et al., 1992) also demonstrated that there was no statistically significant relationship between altitude and genome size. On the other hand, similar to our results for *B. distachyon*, some studies reported a negative correlation between altitude and genome size (Creber et al., 1994; Chia et al., 2012; Manzaneda et al., 2012). However, Guo et al. (2018) reported a positive correlation between altitude and genome size in *Allium* populations.

Table 3. Mean of genome size, standard deviation, and significance group of the studied *B. distachyon* and *B. hybridum* populations from different geographical regions of Turkey.

Species name	Geographical region	Mean of genome size ± SD	Significance group
B. distachyon	Marmara Region (Edirne, Tekirdağ, İstanbul, Çanakkale, Balıkesir)	0.745 ± 0.008	a
	Aegean Region (Kütahya, Muğla)	0.743 ± 0.007	a
	Southeastern Anatolia (Diyarbakır, Adıyaman, Gaziantep, Batman)	0.737 ± 0.011	b
	Central Anatolia Region (Ankara, Eskişehir, Nevşehir)	0.736 ± 0.006	b
MSE = 0.000087			
B. hybridum	Aegean Region (Kütahya, Muğla)	1.435 ± 0.011	
	Mediterranean Region (Antalya, Mersin)	1.428 ± 0.012	
MSE = 0.000366			

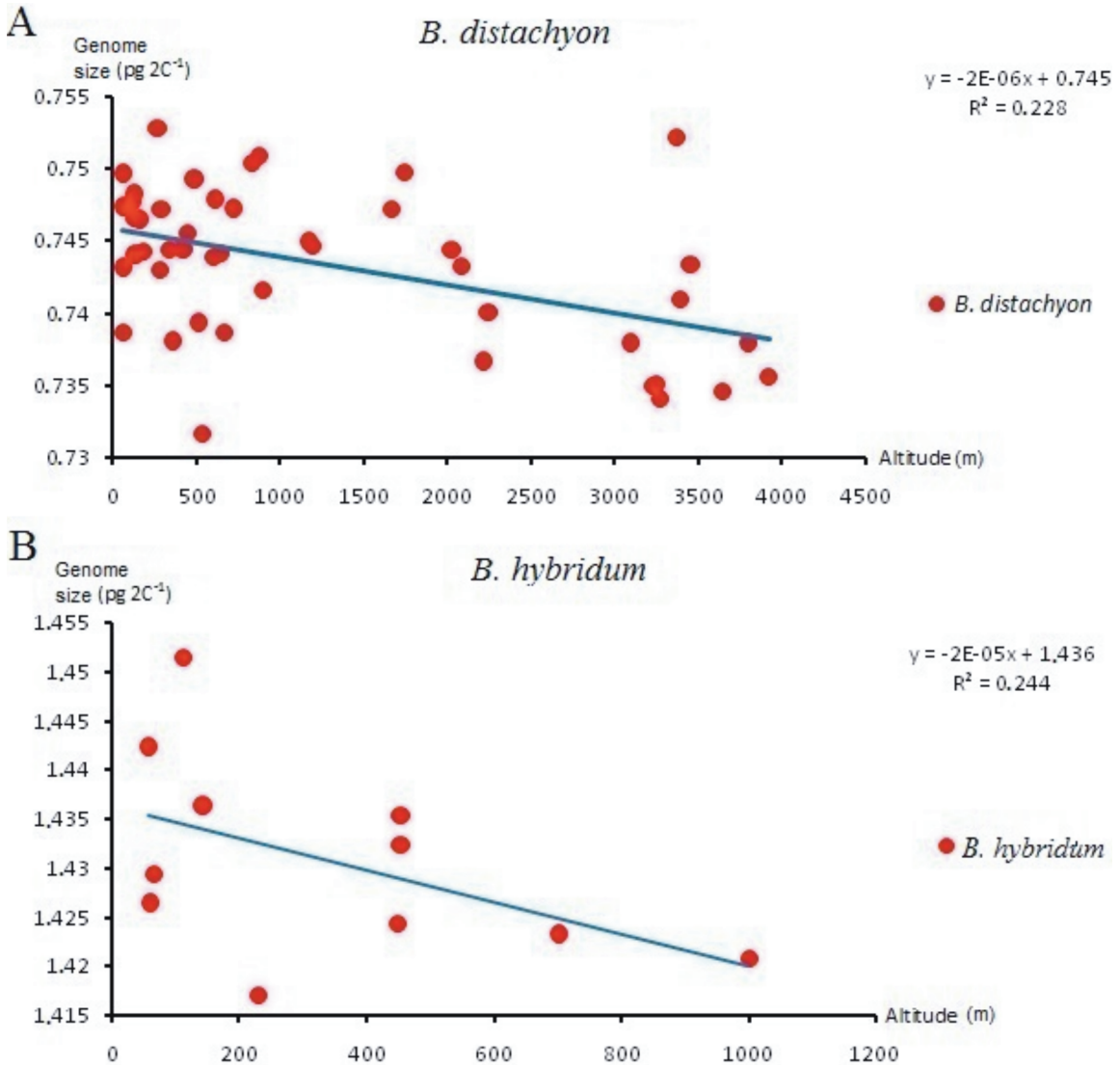

Figure 4. Results of regression analysis between genome size and altitude values; y = the regression equation. A: Based on this analysis, a one-unit increase in altitude (x) caused a statistically significant decrease in nuclear DNA content in *B. distachyon*, B: The same analysis was not significant for *B. hybridum*.

In conclusion, we determined the presence of *B. distachyon* and *B. hybridum* within our collections by flow cytometric analyses, whereas *B. stacei* was not found. Additionally, it was clarified that an intraspecific genome size variation was observed in both *B. distachyon* and *B. hybridum*. Locations, where both species were collected, did not explain the reason for such variation. Moreover, geographical region, geographical distance, and altitude had a statistically significant effect on genome size in *B. distachyon*. In addition, there was a negative correlation between altitude and genome size for *B. distachyon*, whereas these geographical factors were not statistically significant in terms of their effect on genome size in *B. hybridum*. An alternative hypothesis asserts that positive natural selection may indirectly influence genome size variation through developmental or adaptive phenotypes (Knight and Beaulieu, 2008). Connected with this hypothesis, it could be assumed that *B. hybridum* has high ecological tolerance (Scholthof et al., 2018) and it can be considered an adaptive species. It can be hypothesized that natural selection may affect genome size variation in *B. hybridum*. Further studies will be necessary to examine this hypothesis.

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