

Environmental aridity is associated with cytotype segregation and polyploidy occurrence in *Brachypodium distachyon* (Poaceae)

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Summary

- The ecological and adaptive significance of plant polyploidization is not well understood and no clear pattern of association between polyploid frequency and environment has emerged. Climatic factors are expected to predict cytotype distribution. However, the relationship among climate, cytotype distribution and variation of abiotic stress tolerance traits has rarely been examined.
- Here, we use flow cytometry and root-tip squashes to examine the cytotype distribution in the temperate annual grass *Brachypodium distachyon* in 57 natural populations distributed across an aridity gradient in the Iberian Peninsula. We further investigate the link between environmental aridity, ploidy, and variation of drought tolerance and drought avoidance (flowering time) traits.
- Distribution of diploids ($2n = 10$) and allotetraploids ($2n = 30$) in this species is geographically structured throughout its range in the Iberian Peninsula, and is associated with aridity gradients. Importantly, after controlling for geographic and altitudinal effects, the link between aridity and polyploidization occurrence persisted. Water-use efficiency varied between ploidy levels, with tetraploids being more efficient in the use of water than diploids under water-restricted growing conditions.
- Our results indicate that aridity is an important predictor of polyploid occurrence in *B. distachyon*, suggesting a possible adaptive origin of the cytotype segregation.

Introduction

Polyploidy, the addition of whole sets of chromosomes to the genome, is a key process in the evolution of vascular plants (Levin, 2001; Soltis *et al.*, 2004). The origin and magnitude of variation in ploidy level in plants have been studied from different perspectives, including systematics, evolution, reproductive biology, conservation and genetics (recently reviewed in Soltis *et al.*, 2004, 2010; Parisod *et al.*, 2010). However, in contrast to the attention received by the genetic and genomic issues of polyploidization, the ecological and adaptive significance of plant polyploidization remains under-investigated (Soltis *et al.*, 2010).

Numerous studies of spatial distribution of cytotypes of heteroploid species (i.e. species with different ploidy) have documented that polyploids and their lower-ploid progenitors frequently differ in their geographical ranges (reviewed in Levin, 2002; see also e.g. Baack, 2004; Besnard *et al.*, 2008; Kubátová *et al.*, 2008; Balao *et al.*, 2009; Sonnleitner *et al.*, 2010; see however Martin & Husband, 2009). Nonetheless, whether such differences are

attributable to adaptive ecological divergence, or arise from non-adaptive ecological processes (e.g. phylogeographical factors and/or dispersal limitation) is still not well understood (Lumaret *et al.*, 1987; Baack & Stanton, 2005; Buggs & Pannell, 2007; Raabová *et al.*, 2008; Duchoslav *et al.*, 2010; see however Ramsey, 2011), and no clear pattern of association between polyploid occurrence and environment has yet emerged (Buggs & Pannell, 2007). A first approach to investigate the ecological and adaptive significance of ploidal shift is to analyze whether ploidy variations across a species distribution are significantly associated with environmental factors that govern plant species range and abundance. In this sense, examining the relationship between within-species ploidy variation and ecogeographical and climatic variables (Husband & Schemske, 1998; Johnson *et al.*, 2003; Sonnleitner *et al.*, 2010) may be particularly insightful. In particular, climatic factors may be important determinants of differential cytotype distribution at large spatial scales (Treier *et al.*, 2009; Parisod *et al.*, 2010). Some findings support these expectations: polyploids often differ from their diploid progenitors in ecophysiological

tolerance to abiotic stresses (Levin, 2002; see also Buggs & Pannell, 2007; Maherali *et al.*, 2009), and polyploidy is particularly common in habitats affected by climatic fluctuations and/or under extreme climatic conditions (Brochmann *et al.*, 2004; Parisod *et al.*, 2010 and references therein). However, ascertaining accurately which specific physical habitat variables influence cytotype distribution is challenging (Husband & Schemske, 1998). In addition, the presumed relationship between climate and cytotype spatial distribution has rarely been explored empirically through a direct quantitative evaluation of the correlation between the variation in specific climatic parameters and shifts in the frequency of polyploidization, especially at large scales (but see Treier *et al.*, 2009).

Shift in ploidy level is thought to influence differential stress tolerances (Levin, 2002; see also references cited above), which underlies ecological divergence and adaptation of cytotypes to novel environments (Ramsey, 2011). If natural variation of stress tolerance mediated by polyploidization is adaptive, then we should expect a significant association among stress tolerance-related traits and cytotype distribution across a particular stress gradient. This prediction holds regardless of whether ecological divergence of diploids and polyploids arises by ploidal increase itself, or as a result of natural selection operating in polyploid populations. To our knowledge, only one study has previously explored natural variation of abiotic stress tolerance traits in plants from multiple diploid and polyploid populations (Buggs & Pannell, 2007), yet the relationship between such trait variation and the occurrence of polyploidy has not been analyzed across an abiotic stress gradient.

Here, as a part of broader analysis of the ecological and adaptive significance of polyploidy, we use flow cytometry and root-tip squashes to examine the cytotype distribution in the temperate annual grass *Brachypodium distachyon* (Poaceae) in 57 natural populations distributed across a precipitation and aridity gradient in the Iberian Peninsula. In addition, we analyze the link between environmental aridity, ploidy level variation and natural variation of drought tolerance (i.e. water-use efficiency, WUE) and drought avoidance (i.e. time to flowering) traits at a regional scale. Throughout its geographical range, *B. distachyon* shows variation in chromosome number, plants with $2n = 10$, $2n = 20$, and $2n = 30$ chromosomes have been described (Robertson, 1981; Hasterok *et al.*, 2004; Opanowicz *et al.*, 2008), although it is unknown whether such ploidy variation is geographically structured and whether such structure arises in response to environmental cues. We focus here on precipitation, aridity and drought stress tolerance because *B. distachyon* grows in contrasting habitats which are subjected to marked temporal and spatial variations in precipitation and aridity across the Iberian Peninsula and the Mediterranean Basin (Liancourt & Tielbörger, 2009) and significant natural variation in drought tolerance traits has been recently reported for this plant species (Schwartz *et al.*, 2010; Luo *et al.*, 2011), although the putative role of ploidal shifts in drought tolerance has not been investigated. Specifically, we address the following questions. What is the relative frequency of diploids and polyploids in the Iberian *B. distachyon* populations? Is the distribution of each cytotype

geographically structured? After accounting for spatial-geographic autocorrelation, is the distribution of cytotypes significantly associated with variation in precipitation and/or aridity? And is variation in WUE and flowering time contingent on variation in ploidy level?

Materials and Methods

Study species

Brachypodium distachyon (L.) P. Beauv. is a temperate annual small grass (10–20 cm) widely distributed across the Mediterranean Basin, and the Middle and Near East (Catalán & Olmstead, 2000; Opanowicz *et al.*, 2008). In the Iberian Peninsula, *B. distachyon* is widespread, although more abundant in the south and east, and less frequent toward the north and northwest. It grows in a wide variety of habitats under different climatic and ecological conditions (Supporting Information, Table S1), although it is particularly frequent in altered habitats such as roadsides and/or abandoned fields, and in natural xerophytic meadows and forest edges (Table S1).

Brachypodium distachyon has a short life cycle, although its life span varies remarkably among genotypes from different latitudes and elevations (seed to seed cycle time ranges from 6 to 20 wk under long-day laboratory conditions, with 16 h light : 8 h dark; A. J. Manzaneda, unpublished). *B. distachyon* is self-compatible (Catalán & Olmstead, 2000) and seed production, maturation and shedding occur during summer (June–August), but the species may perform either as a winter annual (i.e. germination and seedling emergence occur, without any latency, between September and November) or as a spring annual (i.e. germination occurs in March–April). Germination studies (A. J. Manzaneda & P. J. Rey, unpublished) show that both winter annuals and spring annuals are present in the Iberian Peninsula, but they are not segregated geographically since, independently of germination phenology, winter annuals are clearly overrepresented in our study sites.

Genetic control of flowering time varies notably between winter annuals (which require vernalization before flowering) and spring annuals (which have no vernalization requirement), and also within the winter-annual and spring-annual groups (Schwartz *et al.*, 2010). In any case, flowering occurs naturally between April and June in the Iberian Peninsula populations.

Brachypodium distachyon exhibits variation in somatic chromosome number ($2n = 10, 20, 30$). Polyploid cytotypes with 20–30 chromosomes were initially thought to be tetraploids and hexaploids, originated from a diploid form ($2n = 2x = 10$) by simple multiplication of the same chromosome set (Robertson, 1981). However, detailed comparative cytological analyses using fluorescence *in situ* hybridization (FISH) revealed an allotetraploid origin of all forms with $2n = 30$ (Hasterok *et al.*, 2004). *B. distachyon* diploids ($2n = 10$) may have hybridized with another diploid ($2n = 20$; formerly thought to be autotetraploids, Robertson, 1981) to form the allotetraploid ($2n = 30$) (see Hasterok *et al.*, 2004; Mur *et al.*, 2011 for more details).

Population sampling and climatic data

In June–July 2008, we located and visited 57 natural populations of *B. distachyon* across the Iberian Peninsula. The sampled populations cover most of the ranges of distribution, elevation and types of climate and habitat in which this species grows in the region (Table S1, Fig. 1). The two most distant populations were c. 1119 km apart, whereas the two closest populations were 4 km apart. Populations were located using records from the Spanish Plants Information System database (Anthos, 2008). At each population, we collected 20–100 mature seeds from eight to 20 individual plants chosen randomly within an area of c. 50 × 50 m and separated by at least 1 m from each other. Because diploids and polyploids are indistinguishable in the field, no bias for or against any cytotype is likely in our sample. Seeds were kept in paper envelopes in the laboratory in dark and dry conditions (22°C) until planting.

To characterize each population climatically, we retrieved precipitation (average annual precipitation) and temperature (average annual temperature) data from the geographic information system (GIS)-based Climatic Digital Atlas from the Iberian Peninsula database (Ninyerola *et al.*, 2005; <http://opengis.uab.es/wms/iberia/index.htm>). These data have a spatial resolution of 200 m and include the average values for climatic variables for each month from 1950 to 1999. Selected populations differ notably in both average annual precipitation and average annual temperature (Table S1). From these data we estimated an integrated humidity-aridity parameter as the ratio between average annual rainfall and temperature ($R : T$ ratio hereafter, Table S1). Most of the populations located in the south and southeast of the Iberian Peninsula are subjected to an arid or semiarid Mediterranean climate type, whereas northern populations are located under a humid Eurosiberian climate type (precipitation range: 250–1773 mm; Table S1). A humid or very humid Mediterranean

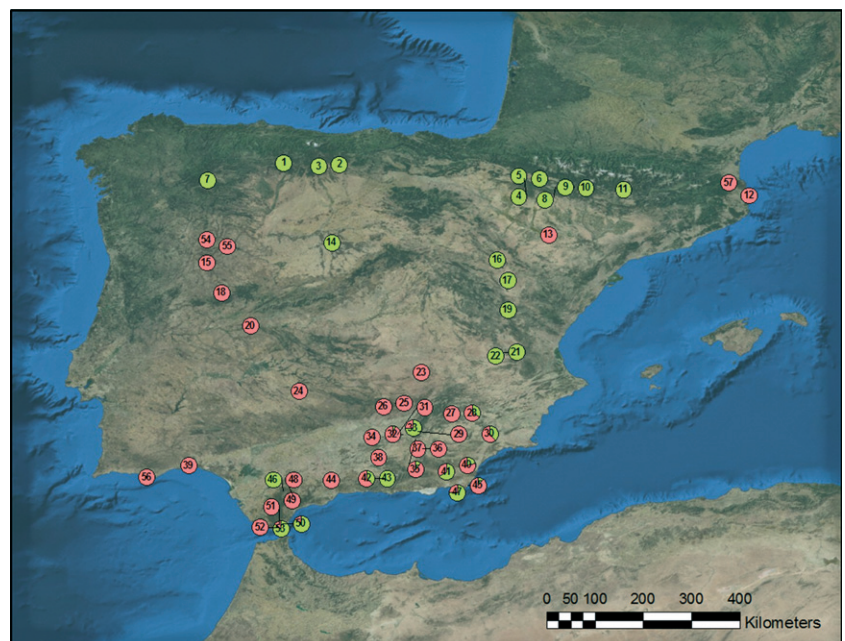
climate type also exists in populations located in the southern mountains and around the Strait of Gibraltar, which constitute a climatic anomaly in southwestern Europe (Table S1; see Ninyerola *et al.*, 2005 for more details on climate of the Iberian Peninsula).

DNA-ploidy determination

Plants were progressively grown in fall 2008 and during 2009 for flow cytometry screening. We germinated two to five seeds per accession (i.e. plant individual lines from our field collection) on moist filter paper in sealed Petri dishes at 4°C for 72 h in dark conditions. Once germinated, seeds were individually planted and grown on standard soil (Fafard 4p mix; Fafard Inc., Agawam, MA, USA) in 48-well flats and maintained in a controlled growth chamber at 21–23°C under long-day conditions (16 h light : 8 h dark).

We used 4,6-diamidino-2-phenylindole (DAPI) flow cytometry on fresh leaves to determine DNA ploidy levels following methods and guidelines as described in Doležal & Bartos (2005) and Suda *et al.* (2007). Data acquisition and fluorescence peak analyses (i.e. G_1 peak means and coefficients of variation (CVs)) were performed using the Flomax[®] FCM software (Münster, Germany). We used fresh leaves from the *B. distachyon* diploid inbred line Bd-21 as an external standard (USDA-ARS NPGS, USA; $2n = 2x = 10$, C -value = 0.44 pg, Vogel *et al.*, 2006). The instrument was adjusted in channel 65 for localizing the G_1 peak of the standard sample. DNA ploidy levels of the samples were then assessed by their mean G_1 peak position relative to the G_1 standard peak. A minimum of 5000 nuclei were analyzed in each sample, and only peaks with $CV \leq 10\%$ were considered in the analyses. To minimize drift effects, standard peaks were obtained at regular intervals (every 10 sample measurements). Each individual plant was replicated twice for a total of 1274 individual plants

Fig. 1 Distribution map of *Brachypodium distachyon* cytotypes in the Iberian Peninsula and geographical origin of the accessions selected in the study. The numbers refer to localities given in Supporting Information (Table S1). The green portion of the pie charts depicts the proportion of diploid (2x) individuals within each population, and the red portion depicts the proportion of tetraploid (4x) individuals. Details on the populations and frequency of polyploids are given in Tables S1 and S2, respectively.



from 637 accessions (between five and 20 accessions per population; mode = 12, see Table S2 for details on sampling effort).

Relationship between relative DNA content and ploidy

To analyze the relationship between DNA content variation and chromosome number, we made chromosome counts on root tips. We germinated seeds from a subsample of 108 accessions from 22 populations (Table S2) on moist filter paper in a sealed Petri dish at a room temperature in dark conditions for 72 h. Once germinated, roots (1–2 cm length) were immersed on ice-cold water for 24 h and thereafter fixed using a (3 : 1) ethanol : glacial acetic acid solution for 24 h at a room temperature. Roots were stained using a 1% solution of acetocarmine for 1 h. Before squashing, root tips were softened into a 5% solution of pectinase, 0.2 g of cellulysin and 0.2 g of cellulose R-10 in a total volume of 20 ml, and incubated for 2 h at 37°C. Finally, root tips were squashed on a microscope slide in 1% acetocarmine and examined under a microscope.

Measurement of drought-tolerance traits

In April 2009 we stratified 288 seeds from 96 different genotypes (A. J. Manzaneda, unpublished) from 24 populations with distinct ploidy levels (diploids vs tetraploids, involving four genotypes per population; Table S3). Populations were selected in order to maximize natural variation in drought-tolerance traits across a significant part of its range in the study region (Fig. 1, Table S3). We minimized potential maternal effects by using seeds from a first generation of self-fertilized, glasshouse-grown plants. Seeds were stratified at 4°C for 1 wk to facilitate uniform seed germination. Plants were grown in 16 h light : 8 h dark at 22°C in 21 cm 'cone-tainers' (Stuewe & Sons Inc., Corvallis, OR, USA) in a randomized complete blocks design, with the 96 genotypes replicated in three blocks. To simulate natural dry-down conditions experienced by the plants in the field after precipitation, plants were watered to saturation at the beginning of the experiment, followed by a 9 wk gradual dry-down treatment (see Knight *et al.*, 2006 for a similar procedure). Under this treatment, plants were watered from above (*c.* 3.8 l per block) just once at the fourth week using a watering can (Fig. S1). Each week we recorded the water content of the soil in the containers at each block (three randomly chosen containers per block) using a volumetric soil moisture TRIME[®]-PICO-32 probe. From the fourth week, soil moisture in the containers declined to values ranging between 10 and 25% until the end of the treatment (Fig. S1). At the fifth week, we moved all plants to a cold room (4°C, 16 h light : 8 h dark) for 3 wk to stimulate flowering in those genotypes which had not yet flowered. This laboratory treatment provides an effective simulation of 'winter' vernalization because plants perceive vernalization cues only when temperatures are above freezing, rather than the long periods of below-freezing temperatures that occur in the wild. Plants were then moved back to the glasshouse until the end of the experiment.

Plants were checked daily once the first plant flowered (day 29) for the onset of flowering (the number of days from germination

until the opening of the first spike). More than 75% of plants had flowered by the ninth week, when we collected one leaf (*c.* 1.6 mg DW) from each plant for isotope analyses. An indicator of WUE integrated over time was determined from the ratio of ¹³C : ¹²C (carbon isotope composition, $\delta^{13}\text{C}$). $\delta^{13}\text{C}$ signatures are positively correlated to WUE in C₃ plant tissues (Farquhar *et al.*, 1989) and have been used recently to investigate interindividual WUE variation in heteroploid species (Buggs & Pannell, 2007). Samples were analyzed for $\delta^{13}\text{C}$ by isotope ratio mass spectrometry at Duke Environmental Stable Isotope Laboratory (<http://www.biology.duke.edu/jackson/devil>). We express WUE values as carbon isotope discrimination ($\Delta^{13}\text{C}$ in ‰) since instantaneous measures of $\Delta^{13}\text{C}$ are straightforward (low $\Delta^{13}\text{C}$ values indicate high WUE) assuming a mean $\delta^{13}\text{C}$ signature of atmospheric CO₂ of – 8.0‰ (see Buggs & Pannell, 2007 for a similar approach).

Data analyses

Independence of geographic distance and cytotype distribution (i.e. between-population Euclidean distance of frequency of polyploids) was tested using a Mantel test (Manly, 1991). The geographic distance matrix was generated by computing the Haversine formula from longitude and latitude data between each population pairwise. Similarly, to investigate the influence of climatic parameters (average annual precipitation and *R* : *T* ratio, i.e. the ratio between average annual rainfall and temperature) on the frequency of polyploids, accounting for spatial-geographic autocorrelation, we conducted partial Mantel's tests (i.e. including a pairwise geographic distance matrix). In this case, separate analyses were performed for precipitation and *R* : *T* ratio pairwise distance matrices. Interrelationships between climatic factors and elevation were detected in our study (Table S4). We attempt to control for such interrelationships by fitting additional partial Mantel's tests examining the correlation of polyploidy frequency with precipitation and *R* : *T* ratio after controlling for elevation differences. Both Mantel's and partial Mantel's statistics were computed after 10 000 permutations performed with the program MantelTester 1.1.

Provided that geographical distance effects were significant (see the 'Results' section), we fitted a binomial multiple regression to further investigate which geographical parameters (latitude, longitude and elevation and their two- and three-way interactions) are correlated with the frequency of polyploids. In this analysis, the frequency of polyploids is estimated from the probability of polyploidy (i.e. the probability of finding a tetraploid cytotype in any population, estimated from the frequency of polyploids/100) given the binomial nature of the frequency of polyploids data distribution. Binomial distribution and logit link function were considered in the model. A Firth bias-corrected maximum likelihood estimator was used to obtain the regression coefficients (Firth, 1993) using JMP 8.0.2 (SAS Institute Inc., Cary, NC, USA).

In addition, we examined the relationship between precipitation, *R* : *T* ratio (although both variables are correlated (see Table S4), to some extent, precipitation may be important *per se* – i.e. without covariation of temperature – for explaining distributional range variations of heteroploid species; e.g. Martin

& Husband, 2009) and the frequency of polyploids. Climatic and geographical data entering this analysis were derived from a single principal component analysis (PCA) conducted to obtain orthogonal factors. The rationale for considering climatic and geographic variables in the same PCA is that both types of variables are frequently interrelated. This may mislead conclusions on climatic correlates of polyploid frequency, which is solved with PCA since it generates truly independent linear combination of variables. Independent climatic and geographic factors were achieved through this analysis, since factor loadings correlated significantly with $R : T$ ratio and precipitation, on the one hand, and with geographical parameters on the other (Table S5). Scores of these two factors and their interaction were subsequently included as independent variables in the binomial regression. Regression coefficients were estimated by Firth bias-corrected maximum likelihood.

To investigate whether carbon isotope discrimination and flowering time vary between ploidy levels, we conducted two separated general linear mixed models with maximum likelihood estimates, using JMP 8.0.2 (SAS Institute Inc.). In these models ploidy was included as a fixed factor, and we considered the influence of population by treating it as a random factor. Flowering time was log-transformed to improve normality and homoscedasticity.

Results

Cytotypes composition and geographical distribution pattern

Flow cytometry analyses revealed the existence of two DNA ploidy levels in our samples (Fig. S2); 40.5% of the analyzed plants were DNA-diploid (median relative DNA content $1 C \pm 1 SD$: 0.448 ± 0.02 , $CV = 5.43\%$), and 59.5% were DNA-tetraploid: 0.827 ± 0.09 , $CV = 10.79\%$). Average fluorescence ratios between the diploid line of *B. distachyon*, used as external standard, and the *B. distachyon* samples were 1.01 and 1.9 for $2x$ and $4x$ cytotypes, respectively (Fig. S3). Chromosome counts performed on 108 plants with different relative DNA content (Table S2) confirmed the presence of just two cytotypes in our samples, which correspond to relative fluorescence values from flow cytometry. Thus, all analyzed DNA-diploids (Table S2) are diploid cytotypes ($2n = 2x = 10$; for simplicity, hereafter 'diploids'), whereas DNA-tetraploids corresponded to $2n = 4x = 30$ allotetraploids (for simplicity, hereafter 'tetraploids') (Table S2). Seven plants from the small area with overlapping DNA-ploidy values (Fig. S2) were cytologically checked, and also corresponded to be $2n = 30$ allotetraploids (Table S2).

Of the 57 populations sampled, 19 (33.33%) contained only diploid individuals, mainly distributed in the north and east-central areas of the Iberian Peninsula (Fig. 1). Uniformly tetraploid populations occurred in 26 sites (45.61%). Although this cytotype was widely distributed, it was more common in the south and west-central region of the Iberian Peninsula (Fig. 1). Mixed populations of diploid and tetraploid cytotypes, at variable frequency, occurred in 12 sites (21.05%). Distribution

of these mixed populations was narrower, and they were located only in the south and southeast of the Iberian Peninsula (Fig. 1).

Dissimilarities in the frequency of polyploids were related significantly to geographical distance between populations ($r = 0.21$, $P < 0.0001$), which indicates that nearby populations have similar frequencies of polyploids. The frequency of polyploids was significantly associated with the three-way interaction of the geographic variables (Table 1), reflecting complex interactions among longitude, latitude and elevation.

Relationship between polyploidy, precipitation and aridity

Differences in the frequency of polyploids were related significantly to dissimilarities in precipitation after controlling for geographical distance (Partial Mantel's statistic, $r_M = 0.072$, $P = 0.0016$) or elevation differences ($r_M = 0.094$, $P = 0.007$). Similarly, differences in the frequency of polyploids were related significantly to dissimilarities in $R : T$ ratio both controlling for geographical distance ($r_M = 0.16$, $P < 0.0001$) or elevation ($r_M = 0.2$, $P < 0.0001$). Therefore, the more similar the precipitation and aridity between populations, the more similar is their frequency of polyploids.

The first and second components of the PCA synthesizing the geographic and climatic data accounted for 70.4% of the variation in the data (Table S5). The first component (PC1) is correlated with precipitation and $R : T$ ratio (Table S5). The second component (PC2) indicates geographical variation, because it is associated with latitude and longitude (Table S5). Both PC1 and PC2 showed a significant negative correlation with the frequency

Table 1 Results of the binomial multiple regression analyzing the relationship between geographical parameters and the frequency of polyploids across 57 *Brachypodium distachyon* populations in the Iberian Peninsula

Source	$\beta \pm 1 SE$	df	χ^2	P
Latitude	-0.43 ± 0.2	1, 49	9.94	0.0016
Longitude	-0.2 ± 0.21	1, 49	4.19	0.0406
Elevation	-0.002 ± 0.001	1, 49	16.77	< 0.0001
Latitude \times longitude	-0.12 ± 0.1	1, 49	6.12	0.0134
Latitude \times elevation	-0.0013 ± 0.005	1, 49	24.06	< 0.0001
Longitude \times elevation	-0.0001 ± 0.0005	1, 49	15.12	0.0001
Latitude \times longitude \times elevation	-0.0003 ± 0.0002	1, 49	18.7	< 0.0001

Significant values ($P < 0.05$) are in bold.

Table 2 Results of the logistic multiple regression analyzing the variation across 57 *Brachypodium distachyon* populations of the probability of polyploidy (the probability of finding a tetraploid cytotype in any population, estimated from the frequency of polyploids/100) as a function of PC1 (which is inversely related to aridity), PC2 (a geographic factor) and their interaction

Source	$\beta \pm 1 SE$	df	χ^2	P
PC1	-0.82 ± 0.31	1, 56	10.13	0.0015
PC2	-0.76 ± 0.34	1, 56	8.74	0.0031
PC1 \times PC2	-0.1 ± 0.35	1, 56	2.21	0.137

Significant values ($P < 0.05$) are in bold.

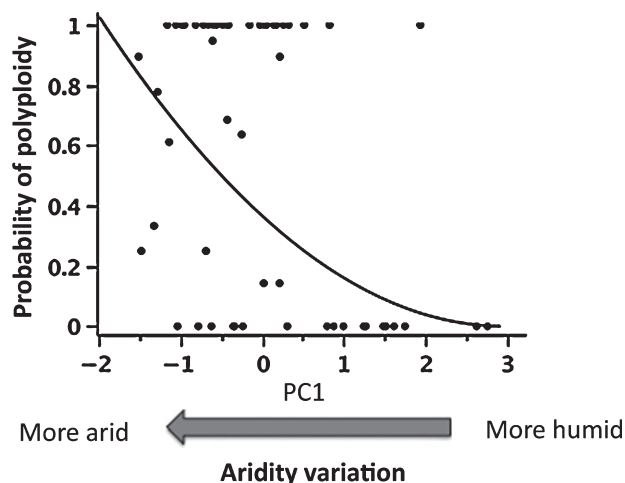


Fig. 2 Variation in the probability of polyploidy (the probability of finding a tetraploid cytotype in any population, estimated from the frequency of polyploids/100) as a function of the PC1 factor, (humidity–aridity variation). Higher and positive values in this factor indicate higher precipitation and lower aridity, because it was positively correlated with both precipitation and the rainfall : temperature ($R : T$) ratio (see the Results section for details). A nonparametric regression (y -square root-transformed) was fitted to the data ($y = (0.61 - 0.2x)^{-1/2}$; $F_{1,56} = 13.66$, $P = 0.0005$; $R^2 = 0.2$).

of polyploids (Table 2). Thus, increases in PC1 (reflecting a more humid environment) were associated significantly with decrements in the frequency of polyploids (Fig. 2). Interestingly, such a relationship was independent of geographical variation across our region, as shown by the nonsignificant interaction between humidity–aridity gradient (PC1) and geographic factor (PC2) (Table 2).

Ploidy and variation in WUE and flowering time

Carbon isotope discrimination ($\Delta^{13}\text{C}$) varied significantly between plants with different ploidy levels ($F_{1,63} = 4.03$, $P = 0.048$). Diploid plants showed significantly higher carbon isotope discrimination than tetraploid plants (Fig. 3). Flowering time ranged between 31 and 154 d after germination, but it did not differ significantly between diploid and tetraploid plants (57.5 ± 0.09 , 52.7 ± 0.09 – Least square means, LSMEANS ± 1 SE values for diploid and tetraploids, respectively; $F_{1,170} = 1.11$, $P = 0.29$).

Discussion

Our study shows interpopulation variation in the ploidy level of *B. distachyon* across the Iberian Peninsula. Distribution of diploid and tetraploid cytotypes in this species is geographically structured across this area, and is associated with aridity and annual precipitation gradients. Moreover, after controlling for geographic and altitudinal effects, the link between aridity and polyploid frequency remains significant. Interestingly, an important stress tolerance trait, WUE, varied between ploidy levels in this species, with tetraploids being more efficient in the use of

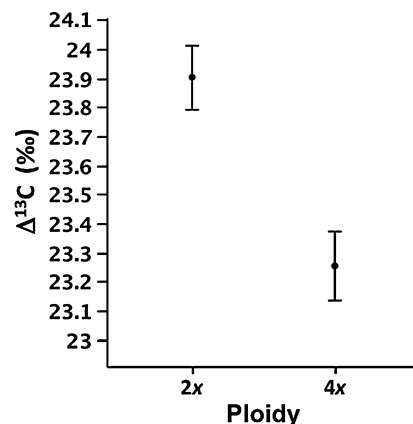


Fig. 3 Variation in carbon isotope discrimination ($\Delta^{13}\text{C}$) between diploids (2x) and tetraploids (4x) *Brachypodium distachyon* plants. Values are adjusted LS-MEANS ± 1 SE.

water than diploids under water-restricted growing conditions. As a whole, our results indicate that aridity may have an important influence on the cytotype segregation and polyploid frequency in *B. distachyon* across the Iberian Peninsula, and suggest that tetraploid cytotypes may better cope with arid conditions through increased tolerance to drought conditions existing in a significant part of the species' range in the Iberian Peninsula. Our results suggest a possible adaptive origin of the cytotype segregation in the *B. distachyon* complex, although other non-adaptive explanations cannot be ruled out.

Cytotype composition and geographical distribution in the Iberian Peninsula

Early karyological studies conducted on material from the Iberian Peninsula and central Europe reported the existence of $2n = 10$, $2n = 20$ and $2n = 30$ cytotypes, with $2n = 30$ being most common (Robertson, 1981). Our data indicate that flow-cytometry tetraploids are the most common cytotype in the Iberian Peninsula (*c.* 60% of the samples analyzed). Such DNA-tetraploids are most likely to be $2n = 30$ since all the tetraploids screened had 30 chromosomes (Table S2). Although we did not perform chromosome counts in all of our populations, our results are consistent with findings that most *B. distachyon* accessions from Eurasia are $2n = 30$ allotetraploids (Wolny *et al.*, 2011). Although we could clearly discriminate diploids from tetraploids (*sensu lato*) by flow cytometry, the relative fluorescence intensity of allotetraploids was twice as high as diploids, rather than the threefold value expected from chromosome counts. Such disparity between karyological and fluorescence data has been reported in some studies (Vogel *et al.*, 2006; Shang *et al.*, 2011) and may suggest that polyploidization was followed by loss of genetic material, a widespread phenomenon in the grass family (Levy & Feldman, 2002). On the other hand, we did not find any triploid intermediate cytotypes, although we detected several mixed populations of diploids and tetraploids. This may reflect prezygotic reproductive isolation between the two cytotypes, and/or a low fertility and viability of triploids within these populations (Husband & Schemske, 1998; Baack, 2005).

Our results show that *B. distachyon* diploid and tetraploid cytotypes are spatially segregated at a regional scale. Thus, although cytotype co-occurrence and mixed populations of diploids and tetraploids exist within the Iberian Peninsula, most populations contain only a single cytotype (c. 80% of the sampled populations consist of either diploids or tetraploids). Diploids are the most common cytotype in the east and north of the Iberian Peninsula, whereas in the south they are either restricted to montane areas at mid- or high elevations, or in populations located near the coast (Fig. 1). By contrast, tetraploids are the most frequent cytotype in the south and west of the Iberian Peninsula at low to mid-elevations, becoming scarcer in the east and north, where they are found primarily at low elevation (Fig. 1). Geographical segregation between diploids and auto- and allopolyploids has been described repeatedly in many species (e.g. Husband & Schemske, 1998; Baack, 2004; see also Levin, 2002 and references therein), including grasses (Keeler, 1990; see also Gibson, 2009).

We detected two contact zones of cytotype coexistence in the Iberian Peninsula, one located at the Strait of Gibraltar, and the other in the southeast (Fig. 1). These two contact zones differ from each other in the relative frequency of diploids and tetraploids present in each population. Mixed populations near the Strait of Gibraltar had a majority of diploids. By contrast, in the southeastern mixed populations, tetraploids and diploids were intermingled, with tetraploids a majority in seven out of 10 populations (Table S2). The existence of contact zones in auto- and allopolyploid complexes seems common, and has also been documented in grasses (reviewed by Petit *et al.*, 1999), and more recently has also been described in some *B. distachyon* populations in northeastern Spain (Mur *et al.*, 2011). Presently, the factors influencing the origin and maintenance of cytotype frequency in these contact zones remain unclear. Experiments to investigate the population dynamics of both cytotypes, habitat differentiation and genotypic selection patterns of diploids and tetraploids under different ecological conditions are ongoing.

Aridity correlates with cytotype distribution through differences in drought tolerance

Divergence in ecological tolerance between polyploids and their diploid ancestors may promote habitat differentiation and spatial separation of cytotypes (Levin, 2002). Our results are consistent with the hypothesis of an adaptive factor in the origin of the geographical separation of the *B. distachyon* cytotypes in the Iberian Peninsula. This support is based on two major findings from this study. First, we found a link between aridity and the frequency of polyploids, with more mesic sites having lower frequencies of tetraploids. Importantly, the association between ploidy variation and aridity and/or precipitation was independent of geographic or altitudinal effects, suggesting that ploidy variation of *B. distachyon* responds, to a large extent, to climatic cues. Secondly, cytotypes differed significantly in their tolerance to drought. Thus, under water-restricted growing conditions, tetraploids were significantly more efficient in their water usage (i.e. lower carbon isotope discrimination) than diploids. The relationship between

aridity and ploidy variation has been previously presupposed (Keeler, 1990) or inferred from other ecological correlates of environmental aridity (Lumaret *et al.*, 1987). Our findings provide functional and quantitative evidence for such positive association at large spatial scales (see also Treier *et al.*, 2009; Duchoslav *et al.*, 2010). Similarly, our results agree with some previous studies which report that polyploids have higher drought tolerance than diploids (reviewed by Levin, 2002; see also Maherali *et al.*, 2009; but see Buggs & Pannell, 2007), and suggest that divergence in drought-tolerance traits between cytotypes is a probable mechanism for segregation of cytotypes across the aridity gradients of the Iberian Peninsula.

Physiological mechanisms underlying the superior drought tolerance of tetraploids go beyond the scope of this work, and will receive more attention in future studies. However, noticeably, divergence in tolerance between cytotypes came from differences in WUE (i.e. dehydration avoidance, McKay *et al.*, 2003), rather than from differences in flowering time (i.e. drought escape). Differences in WUE have previously been shown to underlie habitat differentiation between conspecific or congeneric taxa (Heschel *et al.*, 2002; Savage & Cavender-Bares, 2011), but have seldom been linked to variation in ploidy (but see Buggs & Pannell, 2007). Because WUE measurements were obtained from carbon isotope discrimination values, which essentially reflect tradeoffs between transpiration and carbon fixation (McKay *et al.*, 2003), these differences probably arise from differences in the stomatal apparatus (i.e. density, size and distribution of the leaf stomata) and/or are related to the hydraulic conductivity. Both are known ecophysiological consequences of ploidy changes (Lumaret *et al.*, 1987; Li *et al.*, 1996; Buggs & Pannell, 2007; Maherali *et al.*, 2009).

A question raised by this study is whether the differences between cytotypes in drought tolerance result from genome duplication itself, past interspecific hybridization (Otto, 2007), and/or evolved selectively after polyploidization (Maherali *et al.*, 2009; Ramsey, 2011). It seems likely that tetraploids have a hybrid origin, hence ecogeographical differences and divergence in drought tolerance-related traits may reflect not only effects of ploidal change, but also effects of fixed heterozygosity, genetic novelty resulting from intergenomic recombination, or genetic differences among parental species (Levin, 2002). Future work combining synthetic, newly synthesized allo- and autotetraploids, examination of natural variation across genotypes with different ploidy level, and selection experiments in wild populations will help to answer this question.

Concluding remarks

Although our results agree with the hypothesis of an adaptive origin of cytotype geographic divergence (i.e. divergence in ecological tolerances between diploids and tetraploids), nonadaptive hypotheses for the cytotype distribution exist which have not been ruled out here. First, historical factors and dispersal limitation could contribute to the segregation of diploids and tetraploids (Baack, 2004). Thus, tetraploids may have colonized areas which became available as a result of post-Pleistocene warming,

or after human-induced alterations (e.g. deforestation, fires or agricultural use). The cytotype pattern described here might then reflect the location of earlier cytotype refugia from which colonization by single cytotypes eventually occurred (see Duchoslav *et al.*, 2010 and references therein). Some arguments consistent with these historical hypotheses are that part of the diploid distribution of *B. distachyon* is disjunct in glaciated areas of the Iberian Peninsula (Pyrenees along northern mountains, and Betic mountains in the south, Fig. 1), whereas tetraploids occur in southern and western glacial refugia. Furthermore, *B. distachyon* allopolyploids may have a superior colonizing ability, in accordance with their widespread Eurasian distribution. Finally, long-distance seed dispersal events seem to play a significant role in the genetic population structure of *B. distachyon* at some zones of its geographical distribution (Vogel *et al.*, 2009). Secondly, minority cytotype exclusion (i.e. rare cytotypes will face reduced fitness in mixed populations if reproductive success is frequency-dependent) can lead to spatial segregation of cytotypes (Levin, 2002; Baack, 2004), even if successful self-pollination occurs in polyploids (Buggs & Pannell, 2006).

Our current data are not conclusive in discriminating between these and the adaptive hypothesis. Future work should address demographic aspects (with emphasis on dispersal) of natural populations of each cytotype, along with population genetic studies, to assess the role of demographic and historical factors in the geographical segregation of *B. distachyon* cytotypes in the Iberian Peninsula.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Soil moisture variation during the drought-tolerance experiment.

Fig. S2 Frequency distribution of relative DNA content (pg) obtained from the screening of 1274 individual plants from 637 *Brachypodium distachyon* accessions collected in 57 populations across the Iberian Peninsula.

Fig. S3 Simultaneous flow cytometry analysis of DAPI-stained nuclei isolated from diploid and tetraploid plants.

Table S1 Localities and geographical coordinates of the 57 *Brachypodium distachyon* populations across the Iberian Peninsula used in this study

Table S2 Ploidy level variation among 637 *Brachypodium distachyon* accessions collected from 57 populations in the Iberian Peninsula

Table S3 Location and geographical provenance of the 96 genotypes used in the assessment of the variation in water-use efficiency and flowering time among 24 natural *Brachypodium distachyon* populations across the Iberian Peninsula

Table S4 Spearman's correlation coefficients among the geographical and climate parameters considered in this study

Table S5 Principal component analysis conducted on two climatic and three geographical parameters of 57 *Brachypodium distachyon* populations across the Iberian Peninsula

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