

# Natural variation, differentiation, and genetic trade-offs of ecophysiological traits in response to water limitation in *Brachypodium distachyon* and its descendent allotetraploid *B. hybridum* (Poaceae)

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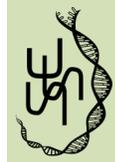
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Differences in tolerance to water stress may underlie ecological divergence of closely related ploidy lineages. However, the mechanistic basis of physiological variation governing ecogeographical cytotype segregation is not well understood. Here, using *Brachypodium distachyon* and its derived allotetraploid *B. hybridum* as model, we test the hypothesis that, for heteroploid annuals, ecological divergence of polyploids in drier environments is based on trait differentiation enabling drought escape. We demonstrate that under water limitation allotetraploids maintain higher photosynthesis and stomatal conductance and show earlier flowering than diploids, concordant with a drought-escape strategy to cope with water stress. Increased heterozygosity and greater genetic variability and plasticity of polyploids could confer a superior adaptive capability. Consistent with these predictions, we document (1) greater standing within-population genetic variation in water-use efficiency (WUE) and flowering time in allotetraploids, and (2) the existence of (nonlinear) environmental clines in physiology across allotetraploid populations. Increased gas exchange and diminished WUE occurred at the driest end of the gradient, consistent with a drought-escape strategy. Finally, we found that allotetraploids showed weaker genetic correlations than diploids congruous with the expectation of relaxed pleiotropic constraints in polyploids. Our results suggest evolutionary divergence of ecophysiological traits in each ploidy lineage.

**KEY WORDS:** Adaptation, flowering time, gas exchange, genetic correlations, hybridization, polyploidy.

Polyploidy (i.e., whole-genome duplication) alters DNA content, chromosome number and organization, and gene dosage (Soltis et al. 2004), and has been a pivotal feature of vascular plant diversification (Wood et al. 2009; Jiao et al. 2011). Newly formed

polyploid lineages often differ in ecological tolerances and phenotypes from their progenitors (e.g., Chao et al. 2013), which may lead to population divergence and ecological speciation (Levin 2002; Ramsey 2011; Martin and Husband 2013). Polyploidy is a



recurrent form of genetic variation (Levin 2002; Parisod 2012), yet we know little about how ploidy influences standing natural variation in ecologically important traits (see, however, Nuismer and Cunningham 2005; Oswald and Nuismer 2010; Martin and Husband 2012).

In habitats worldwide, water stress influences plant adaptation, abundance, distribution, and productivity (Heschel et al. 2002; McKay et al. 2003; Engelbrecht et al. 2007). Plants have evolved several strategies to cope with drought stress, including dehydration avoidance, drought tolerance, and drought escape by reproducing before the onset of drought (McKay et al. 2003; Sherrard and Maherali 2006; Tuberosa 2012). These strategies rely on complex ecophysiological traits whose effects may vary across environments (e.g., Ehrenreich et al. 2009; Xu et al. 2009; Tardieu and Tuberosa 2010). In dry environments, drought-avoiding  $C_3$  plants have evolved elevated water-use efficiency (WUE) by closing stomata, which restricts transpiration more than it restricts photosynthesis. This trade-off between  $CO_2$  uptake and water loss depends on stomatal anatomy and conductance, which may be targets of selection (Ackerly et al. 2000; Heschel et al. 2002; Donovan et al. 2007; Agrawal et al. 2008). Alternatively, plants with a drought-escape strategy maintain rapid growth, higher gas exchange, and early flowering, allowing the plant to complete its life cycle before the onset of water limitation (Heschel and Riginos 2005; Sherrard and Maherali 2006; Donovan et al. 2007; Wu et al. 2010). Ecophysiological traits associated with drought tolerance and escape vary genetically within and among populations (Ackerly et al. 2000; Geber and Griffen 2003; Caruso et al. 2005; Culley et al. 2006; Sherrard et al. 2009), which could lead to adaptive population divergence if selection differs across contrasting environments (Ackerly et al. 2000; Donovan et al. 2009). However, adaptive evolution of ecophysiological traits could be constrained if traits are genetically correlated and the sign of the correlations conflicts with the magnitude and direction of selection on the traits (e.g., McKay et al. 2003; Caruso et al. 2005; Sherrard and Maherali 2006); or if expression of genetic covariation depends upon water availability (i.e., genetic trade-offs across environments; Sherrard et al. 2009; Ivey and Carr 2012).

In heteroploid species, ecological tolerance to water limitation may differ between polyploids and their lower-ploidy ancestors due to morphological and physiological changes derived from polyploidization (Levin 2002; te Beest et al. 2012). In perennial species, polyploid lineages often have greater physiological capability to respond to water deficit than their diploid progenitors through (1) increasing WUE and reducing stomatal conductance, transpiration, and growth rate and (2) maximizing water uptake by optimizing plant hydraulic function (Li et al. 1996; Maherali et al. 2009; Allario et al. 2013; Hao et al. 2013). This dehydration-avoidance strategy of polyploids may allow them to differentiate

in drier environments than diploids (e.g., Treier et al. 2009; Hao et al. 2013). Alternatively, for heteroploid annuals growing across aridity gradients, ecological differentiation of polyploids in drier environments could be based on a drought-escape strategy involving high gas exchange, low WUE, rapid development, and early flowering; this functional strategy is often favored under drought stress in annual species (Heschel and Riginos 2005; Sherrard and Maherali 2006; Donovan et al. 2007; Ivey and Carr 2012). Polyploids that employ a drought-escape strategy would show positive relationships among photosynthesis, stomatal conductance, and aridity, and negative relationships among WUE, flowering time, and aridity.

The ability of polyploids to colonize new habitats outside of the range(s) of the diploid progenitor may depend on the extent of genetic variation and plasticity in ecophysiology and other key traits (Levin 2002; Hahn et al. 2012; te Beest et al. 2012). Polyploids may show greater genetic variability and increased plasticity compared with diploids because of increased heterozygosity (especially in allopolyploids formed by interspecific hybridization, Doyle et al. 2008), which in turn can confer a higher adaptive response to environmental change (Soltis et al. 2010; Hahn et al. 2012; te Beest et al. 2012; Madlung 2013). Furthermore, increased gene copy number in polyploids could ease pleiotropic constraints (Otto 2007), and enhance the capacity of polyploids to adapt in response to novel selection.

Here, we use the diploid annual grass *Brachypodium distachyon* and its derived allotetraploid *B. hybridum* (Poaceae) to examine natural variation and differentiation in ecophysiological traits in diploid ( $2n = 10$ ) and allotetraploid ( $2n = 30$ ) accessions from multiple wild populations across the Iberian Peninsula. *Brachypodium* ploidies are geographical and ecologically differentiated (Manzaneda et al. 2012). Both lineages grow in Mediterranean-type climate, which is typically characterized by a relative long estival drought. However, *B. distachyon* diploids are found frequently in wet habitats in which summer drought is attenuated; therefore, a drought-escape strategy might not be adaptive (but see Sherrard and Maherali 2006). In contrast, *B. hybridum* allotetraploids inhabit mainly dry environments in which a predictable summer drought period exists, and a drought-escape strategy could confer fitness advantages, as is true for other winter annual grasses in Mediterranean climates with prolonged dry periods (e.g., Sherrard and Maherali 2006 and references therein). In addition, *B. hybridum* allotetraploids have larger stomata (Catalán et al. 2012) and are more efficient in their water use than diploids (Manzaneda et al. 2012). Although high WUE is typically associated with a dehydration-avoidance strategy, increased WUE could be advantageous in water-limited environments if it enhances the likelihood of survival to reproduction in a shortened growing period (Wu et al. 2010). Likewise, accessions of this species show significant variation in flowering

time (Schwartz et al. 2010), although the adaptive significance of such variation is unknown. The overarching objectives of this study were to evaluate whether physiological differences among *Brachypodium* ploidy levels underlie observed ecogeographical differentiation (Manzaneda et al. 2012), and to analyze the potential for adaptive evolution of different *Brachypodium* ploidy lineages in populations with contrasting aridity.

By exposing multiple accessions of these lineages to two contrasting watering regimens in the greenhouse, we tested whether *Brachypodium* ploidy levels differ in gas exchange physiology and flowering times. We predict that under water limitation, allotetraploids will maintain higher photosynthesis and stomatal conductance and flower earlier than diploids, consistent with a drought-escape strategy. We then analyzed the extent of broad-sense genetic variation within and between populations and plasticity in these traits for each ploidy level, to test the prediction that allotetraploids maintain higher genetic variation and increased plasticity than diploids. We also tested for associations between trait variation and aridity at the level of the population. In particular, we hypothesize that allotetraploid lines from warmer, drier populations will have higher photosynthesis, stomatal conductance and earlier flowering than lines from cooler and wetter populations. Finally, we examined genetic correlations among ecophysiological traits under contrasting water conditions in diploid and allotetraploid lines to identify potential evolutionary constraints to adaptation.

## Materials and Methods

### STUDY SYSTEM

*Brachypodium distachyon* (L.) P. Beauv. is a temperate annual grass (10–20 cm) native to the Mediterranean Basin, Middle and Near East (Catalán et al. 2012). This grass inhabits a wide variety of climatic and ecological conditions from sea level to 2000 m elevation; it is frequently found in forest edges, natural xerophytic meadows, abandoned fields, and along roadsides (Manzaneda et al. 2012). This self-compatible grass (heterozygosity ranges between 0 and 0.016; Vogel et al. 2009) has a short life-cycle, and germination studies show that both winter-annuals and spring-annuals are present in the Iberian Peninsula (Manzaneda and Rey, unpubl. ms.). Flowering time varies notably within and between winter-annuals (which require vernalization before flowering) and spring-annuals (which have no vernalization requirement) in response to temperature and light cues (Schwartz et al. 2010). Similarly, *B. hybridum* allotetraploid genotypes typically do not require vernalization to flower (Martínez and Manzaneda, unpubl. ms.). Flowering occurs naturally between April and June in the Iberian populations and seed maturation and dispersal occur during June to August.

The *B. distachyon* complex exhibits variation in somatic chromosome number ( $2n = 10, 20, 30$ ; Robertson 1981). Fluores-

cence in situ hybridization (FISH and GISH) techniques revealed that  $2n = 30$  is an allotetraploid derived from two diploids ( $2n = 10$  and  $2n = 20$ , Hasterok et al. 2004), and Catalán et al. (2012) recently proposed that these three cytotypes are in fact three closely related species: two diploids with a different chromosome base number, *B. distachyon* ( $x = 5, 2n = 10$ ) and *B. stacei* ( $x = 10, 2n = 20$ ), and their derived allotetraploid *B. hybridum* ( $x = 5 + 10, 2n = 30$ ). In the Iberian Peninsula, the two most common cytotypes are the diploid *B. distachyon* and allotetraploid *B. hybridum* whereas populations of *B. stacei* have been found just in a few localities in southeast Spain (Manzaneda et al. 2012; López-Alvarez et al. 2012). *Brachypodium distachyon* diploids (“diploids” hereafter) are the most common cytotype in the east and north of the Iberian Peninsula, whereas *B. hybridum* allotetraploids (“allotetraploids” hereafter) are the most frequent cytotype in the south and west of the Iberian Peninsula at low-to-middle elevations (Manzaneda et al. 2012). Independent of geography, allotetraploids are found in drier locations than diploids (Manzaneda et al. 2012).

### PLANT MATERIAL AND EXPERIMENTAL PROCEDURE

We selected mature seeds from 189 accessions (i.e., 90 diploid and 99 allotetraploid inbred lines derived from our field collection) from 24 populations (about eight lines/population) distributed across the Iberian Peninsula (Tables S1, S2; Fig. S1). Ploidy level of each line was previously determined using flow cytometry and root-tip squashes (Manzaneda et al. 2012). These populations are found in diverse climatic conditions. We chose these populations to maximize natural variation in drought-tolerance traits across a significant part of the range in the study region (Table S1). Nine populations contain uniformly diploid plants, ten populations contain purely allotetraploids, and five are mixed populations of diploids and allotetraploids (Tables S1, S2).

In April 2009, we stratified eight sib seeds of each selfing-inbred line ( $N = 1512$  seeds total) at 4°C for one week to facilitate uniform seed germination. We minimized potential maternal effects by using seeds from a first generation of self-fertilized, greenhouse-grown plants. Plants were grown on standard soil (Fafard 4p mix; Fafard, Agawam, MA) at the Duke University greenhouse in 16-h light: 8-h dark at 22°C in 21-cm “conetainers” (Model SC10R, volume: 164 mL; Stuewe & Sons, Corvallis, OR) in a split-plot design with watering treatment as the whole plot factor. Specifically, plants were exposed in two separate benches (distance between benches was about 1 m) to one of two watering treatments: well watered or water restricted. Each line (189 in total) was replicated four times in separate blocks for each watering treatment (within each block, plants were planted randomly). In the well-watered treatment, plants were kept watered by using bottom watering trays over nine weeks. In the water-restricted treatment, we simulated natural dry-down conditions experienced by plants in the field after precipitation. Initially, plants were

watered to saturation, followed by a nine-week gradual dry-down treatment during which time we watered plants from above (~3.8 L/block) just once during the fourth week (Fig. S2). Weekly, we used a volumetric soil moisture probe (TRIME-PICO-32) to record water content of the soil of three randomly chosen containers per block. In the well-watered treatment, soil moisture was always above 65%, whereas under water restriction soil moisture averaged  $45 \pm 6.4\%$  (mean  $\pm$  SE), although from the fourth week until the end of the experiment it declined to 20 to 25% (ANOVA on soil moisture variation, water treatment  $\times$  date interaction:  $F_{1,8} = 12.63$ ,  $P = 1.66^{-10}$ ; Fig. S2). Tissue damage increases due to wilting at soil moisture levels  $<20\%$ , often producing unreliable measurements of gas exchange (i.e., negative gas exchange values and fluctuation of gas exchange curves). For that reason, we set 20% soil moisture (slightly above the wilting point) as the minimum threshold for obtaining reliable physiological data from our plants. The drought treatment had an effective impact on plant physiology (see Results) and development (Fig. S3).

#### ECOPHYSIOLOGY AND FLOWERING PHENOLOGY

After three weeks of plant growth, we measured physiology of 1161 plants (351 plants died before that point). We quantified whole-plant photosynthesis (i.e., carbon gain,  $A$ ) and stomatal conductance ( $g_s$ ) with an infrared gas analyzer (IRGA, LI-COR LI-6400 portable photosynthesis system) modified to fit a set of four whole-plant cuvettes in a parallel processing system (see Fig. S4 and Supporting Information for methodological details).

We estimated WUE integrated over the lifetime of the leaf from the ratio  $^{13}\text{C}/^{12}\text{C}$  (carbon isotope composition,  $\delta^{13}\text{C}$ ; e.g., McKay et al. 2003; Sherrard and Maherali 2006; Chen et al. 2011). At the 10th week of the experiment, we collected one leaf per plant for isotope analysis from 95 lines: 48 diploids and 47 allotetraploids, replicated three times in each water treatment. Samples were analyzed by isotope ratio mass spectrometry at Duke Environmental Stable Isotope Laboratory ([www.biology.duke.edu/jackson/devil](http://www.biology.duke.edu/jackson/devil)).

Some genotypes require a cold vernalization period to induce flowering (mainly *B. distachyon* diploid genotypes), after taking gas exchange measurements, thus we moved plants to a cold room (4°C, 16-h light and 8-h dark, about three weeks; Fig. S2). To ensure that individuals experienced the same environmental conditions across the experiment, we moved all plants into the cold room whether they had flowered or not. Plants were then moved back to the greenhouse until the end of the experiment. To quantify flowering time we monitored plants daily once the first plant flowered (after about four weeks from germination) for about 150 days, after which point no remaining plants flowered. We defined flowering time as the number of days from germination until the opening of the first spike.

#### STATISTICAL ANALYSIS

To test differentiation in ecophysiological traits between ploidy and treatments, we conducted general linear mixed models (GLMM) with ploidy, watering treatment and their interaction as fixed effects. We examined the “ploidy  $\times$  treatment” interaction by conducting tests of simple main effects using the SLICE option in SAS (ver. 9.3, SAS Institute). In these models, we treated line nested in ploidy and block as random effects. To incorporate the split plot for watering main effect testing, we nested the random block effect within watering treatment. By this way, degrees of freedom (df) for assessing the effect of the watering treatment come from the set of individuals in each block. We included the number of spikes as a covariate in the models because the presence of inflorescences may influence gas exchange (Earley et al. 2009).

We analyzed flowering time by fitting a proportional hazards Cox regression model (Proc PHREG, SAS). To account for the effect of differences among lines and blocks in flowering time, line was incorporated as a random effect (i.e., “frailty”) and block as a fixed effect (due to limitations of the statistical package only one random factor can be included in the same model).

We estimated the degree of phenotypic plasticity for each trait and ploidy level from the difference in the cytotype least squares adjusted means between the two watering treatments. Significance of plasticity values comes from interactions’ slice tests and contrast tests. In addition, for traits with significant plasticity, we analyzed genetic variation in phenotypic plasticity. Due to model convergence issues, we opted to fit separate GLMM to investigate variation in plasticity across lines in each ploidy level. In these models, population was considered a fixed factor, and line nested within population and block as random effects.

To analyze broad-sense genetic variation in ecophysiology within and among populations, we first fit GLMM with restricted maximum-likelihood estimates using Proc MIXED, with population as a fixed factor and random effects for line nested within population and block. Statistical inferences (Wald’s  $Z$  test) for the covariance parameters for random factors were computed using the COVTEST statement in the procedure MIXED. We fit separate models for each combination of trait, ploidy level, and watering regime. Similarly, we obtained the coefficients of variation for each trait. Before and during ecophysiological measurements, 443 plants began flowering. For that reason, we also included the number of spikes produced per each plant at the time of the gas exchange measurements as a covariate in models of whole-plant gas exchange variation. Nonflowering individuals (380 plants) were not included in the analyses to estimate genetic variation in flowering time.

We also analyzed the relative contribution of line and population to the total phenotypic variance. We conducted a variance decomposition following a hierarchical design and using restricted

**Table 1.** Summary results of general linear mixed models testing the effects of ploidy (P), water treatment (T), and their interaction on variation of ecophysiological traits across multiple *Brachypodium distachyon* diploid and *B. hybridum* allotetraploid lines.

Source	<i>A</i> (μmol CO <sub>2</sub> m <sup>-2</sup> s <sup>-1</sup> )			<i>g<sub>s</sub></i> (mmol m <sup>-2</sup> s <sup>-1</sup> )			Integrated WUE (δ <sup>13</sup> C)		
	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>
Ploidy (P)	1,169	0.08	0.78	1,165	8.79	<b>0.0035</b>	1,93	8.15	<b>0.0053</b>
Water treatment (T)	1,6.21	7.21	<b>0.0351</b>	1,6.24	73.98	<b>0.0001</b>	1,4.28	182.72	<b>0.0001</b>
P × T	1,981	36.84	<b>&lt;0.0001</b>	1,977	2.63	0.105	1,414	1.13	0.289
Random effects		<i>Z</i>	<i>P</i>		<i>Z</i>	<i>P</i>		<i>Z</i>	<i>P</i>
Line (ploidy)		5.38	<b>&lt;0.0001</b>		4.89	<b>&lt;0.0001</b>		4.86	<b>&lt;0.0001</b>
Covariate	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>
Number of spikes	1,1006	78.93	<b>&lt;0.0001</b>	1,876	125.77	<b>&lt;0.0001</b>	NA	NA	NA

The effect of “line” nested within ploidy was incorporated as a random factor in the models. We included the number of spikes as a covariate in gas exchange models. Significant values (*P* < 0.05) are in bold.

maximum-likelihood estimates (that account for unbalanced data) in Proc MIXED models that treated population and line (nested within population) as random factors. We compared the fraction of total phenotypic variance for each trait among lines within population (*V<sub>line</sub>*) and among populations (*V<sub>pop</sub>*), with the residual variance (*V<sub>res</sub>*) corresponding to the variance between blocks and individuals within line. We tested whether these variance components differ significantly from zero using a likelihood ratio test with 1 df (e.g., Colautti and Barrett 2011).

For traits with significant genetic variation, we investigated the relationship between trait variation and climate. We used trait population means instead of trait genotypic means to avoid pseudoreplication (see Maron et al. 2007 for a similar procedure). We then analyzed the relationship among population mean trait values and precipitation, temperature, and soil moisture deficit separately for each trait and ploidy level. Across our study populations, water availability is not linearly correlated with latitude and longitude (Fig. S5); therefore, we conducted both linear and nonlinear regressions. More details on climate correlates are given in Supporting Information.

To estimate potential genetic constraints and limits to adaptive evolution for each ploidy lineage, we estimated genetic correlations across individuals of each ploidy level based on variance components from REML (Proc Mixed, SAS) using individual-level data among all trait pairs. Genetic correlations computed from a randomly chosen population may be a poor predictor of constraints on adaptive evolution across the range of a species (Sgrò and Hoffmann 2004; Colautti and Barrett 2011). Thus, by including the entire range of trait variation from all populations, we analyzed genetic trait (co)variance in a dataset with maximum range of genetic variation for each trait. Genetic correlations were calculated as follows:

$$r_g = \text{Cov}(i, j) / \sigma_i \sigma_j,$$

where *Cov*(*i*, *j*) is the genetic covariance component for traits *i* and *j*, and  $\sigma_i$  and  $\sigma_j$  are the square roots of the among-genotype

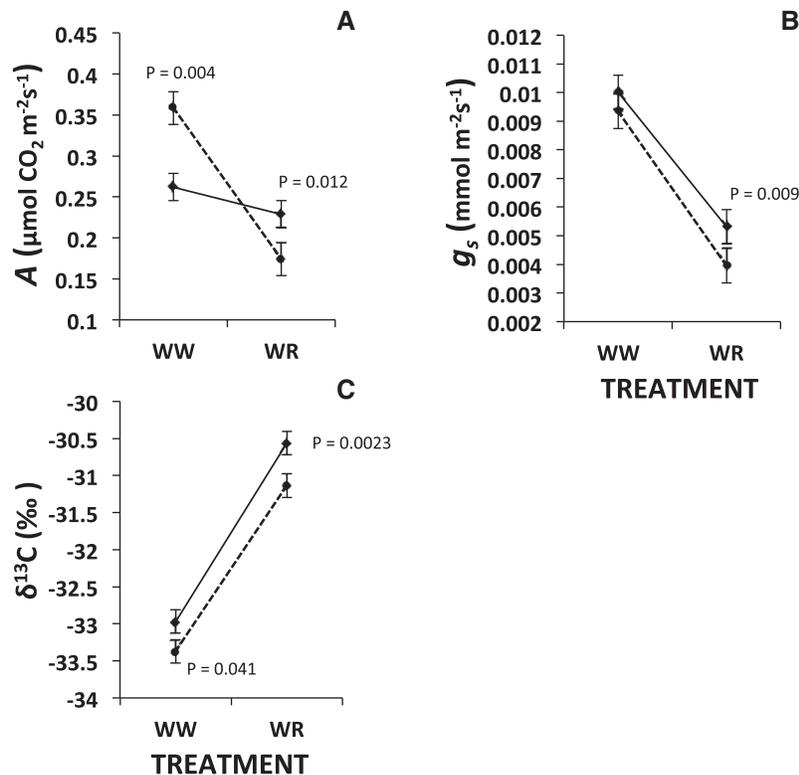
variance components for traits *i* and *j* (e.g., McKay et al. 2003). Significance was determined by log-likelihood ratios tests comparing full models with reduced models in which the covariance was constrained to 0. We used the full dataset to estimate correlations among whole-plant gas exchange traits and flowering times on all plants. We used the subset of individuals for which δ<sup>13</sup>C had been determined to estimate correlations among this trait and the rest of traits. All correlations were computed from both treatments because genetic correlations may differ across environments (Sgrò and Hoffmann 2004; Sherrad et al. 2009). Finally, we employed Cheverud’s random skewers method (Cheverud and Marroig 2007) to quantitatively compare genetic variance–covariance matrices among ploidy lineages within treatment, or between treatments within each ploidy lineage. If the mean response cosine is larger than 95% of the cosine of random vectors, then we cannot reject the null hypothesis that the two G-matrices are equal or proportional. See Supporting Information for further details on this procedure. For all analyses, photosynthesis and stomatal conductance were log-transformed to improve normality and homoscedasticity.

## Results

### TRAIT DIFFERENTIATION

#### Ecophysiological traits

The effect of watering on photosynthesis differed for each ploidy level (i.e., significant ploidy × treatment; Table 1). Under well-watered conditions, diploids showed significantly higher whole-plant carbon gain than allotetraploids (Fig. 1A). In contrast, under water-restricted conditions, diploids had significantly lower carbon gain than allotetraploids (Fig. 1A). Ploidy significantly affected stomatal conductance and δ<sup>13</sup>C (Table 1). Allotetraploids had higher stomatal conductance and WUE (higher δ<sup>13</sup>C) than diploids (Fig. 1B, C), especially under water-restricted treatment (Fig. 1B, C). In all traits, there was significant among-line variation within each ploidy level (Table 1). Finally, the number of



**Figure 1.** Variation (LS-MEANS adjusted model values  $\pm 1$  SE) in (A) whole-plant photosynthesis (A), (B) stomatal conductance ( $g_s$ ), (C) carbon isotope composition ( $\delta^{13}\text{C}$  ‰) between two watering treatments and two levels of ploidy variation: *Brachypodium distachyon* diploids (dashed line) and *B. hybridum* allotetraploids (solid line). *P*-values in the graphs are results from test of the slice interactions comparing both ploidy levels in each treatment. Only results from significant tests are shown. WW, well watered; WR, water restricted.

spikes was positively associated with whole carbon gain and stomatal conductance ( $0.035 \pm 0.005$ ,  $0.065 \pm 0.005$ ,  $b \pm 1$  SE for A and  $g_s$ , respectively).

#### Flowering phenology

During the experiment 70.9% of plants ( $N = 1307$ ) flowered. The effect of water level depended on ploidy (ploidy  $\times$  treatment likelihood ratio test:  $\chi^2 = 5.77$ ,  $df = 1$ ,  $P = 0.016$ ; treatment:  $\chi^2 = 8.43$ ,  $df = 1$ ,  $P = 0.0036$ ; ploidy:  $\chi^2 = 0.322$ ,  $df = 1$ ,  $P = 0.57$ ). Under well-watered conditions, diploids and allotetraploids had essentially the same probability of flowering through time ( $df = 1$ ,  $\chi^2 = 2.05$ ,  $P = 0.152$ ; Fig. 2). However, under water restriction diploids had a 34.6% lower probability of flowering than allotetraploids ( $df = 1$ ,  $\chi^2 = 7.75$ ,  $P = 0.0054$ ; Hazard Ratio 0.654, 95% CL: 0.528–0.812; Fig. 2).

#### PHENOTYPIC PLASTICITY

Both diploids and allotetraploids showed significant plasticity to watering treatment for all traits analyzed (Table S3, Fig. 1). Plasticity varied among ploidies only for carbon gain, with diploids showing higher plasticity in this trait (ANOVA:  $F_{1,164} = 13.17$ ,  $P = 0.0004$ ; Table S3).

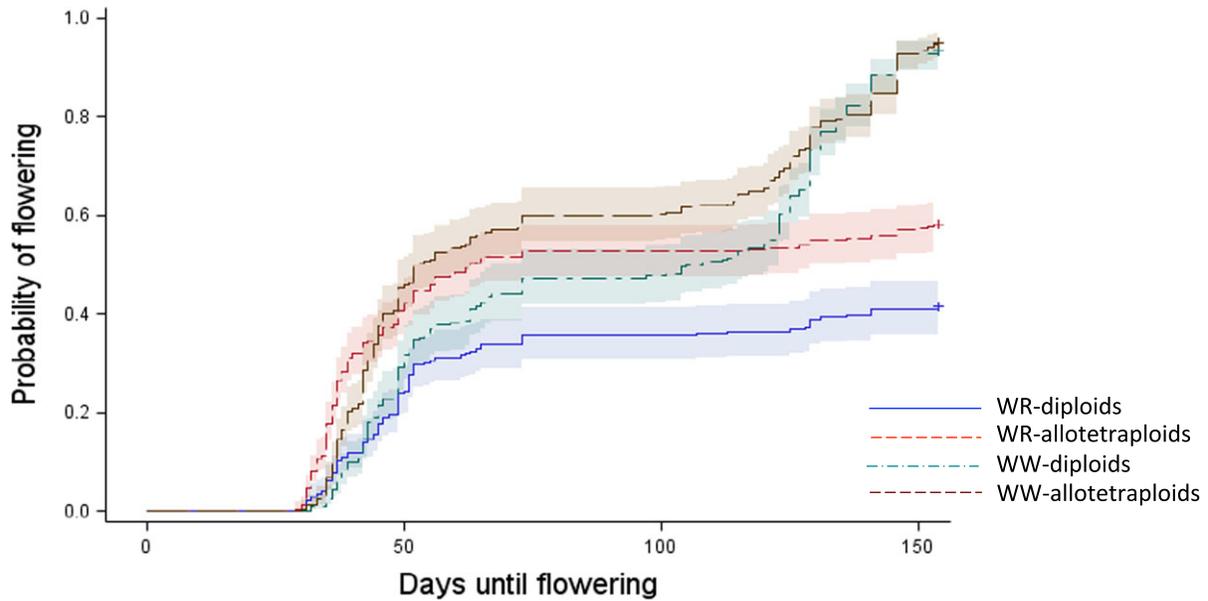
For diploids, there was significant genetic variation in plasticity among populations in all traits (Table 2). In addition,

$g_s$  plasticity also varied among lines within population (Table 2). Plasticity of allotetraploids significantly differed at population level for  $g_s$  and flowering time (Table 2).

#### EXTENT OF GENETIC VARIATION

We found extensive genetic variation in physiology and flowering time, although in most cases population rather than lines within population accounted for the genetic variation in these traits (Table 3; Figs. S6, S7). With the exception of  $g_s$ , trait variation was higher under water restriction than in well-watered conditions (Table 3). Under well-watered conditions, allotetraploids showed higher trait variation than diploids in photosynthesis and flowering time, similar variation in  $\delta^{13}\text{C}$ , and lower variation in  $g_s$ . Under water restriction, allotetraploids showed higher levels of variation than diploids in flowering time,  $\delta^{13}\text{C}$ , similar in  $g_s$ , and lower in photosynthesis (Table 3). In both ploidies, the number of spikes was positively associated with whole carbon gain and  $g_s$  (Table S4) under both watering treatments.

For diploids, trait variance was greater among populations than among lines within population in both treatments (Table 4). For allotetraploids, whole carbon gain,  $g_s$  and flowering time exhibited significantly greater phenotypic variance among populations than genetic variance within populations, but the



**Figure 2.** The probability of flowering for *Brachypodium distachyon* diploid and *B. hybridum* allotetraploid individuals in each watering treatment as a function of time (number of days). Shading indicates 95% confidence intervals.

**Table 2.** Summary results of the general linear mixed analyses conducted to test for genetic variation at population and family levels (i.e., significance of factor “line”) in plasticity of physiological traits and flowering time across *Brachypodium distachyon* diploid and *B. hybridum* allotetraploid lines.

Diploids	Population			Line (population)	
Traits	df	F	P	Z	P
A ( $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ )	13,77	2.26	<b>0.014</b>	0.45	0.651
$g_s$ ( $\text{mmol m}^{-2}\text{s}^{-1}$ )	13,59	8.77	<b>&lt;0.0001</b>	1.99	<b>0.046</b>
Integrated WUE ( $\delta^{13}\text{C} \text{ ‰}$ )	13,33	3.64	<b>0.001</b>	0.77	0.44
Flowering time (no. of days)	13,79	2.2	<b>0.016</b>	1.07	0.285
<b>Allotetraploids</b>					
A ( $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ )	14,72	0.51	0.917	1.03	0.301
$g_s$ ( $\text{mmol m}^{-2}\text{s}^{-1}$ )	14,87	2.31	<b>0.0095</b>	0.2	0.838
Integrated WUE ( $\delta^{13}\text{C} \text{ ‰}$ )	14,34	1.17	0.334	1.34	0.181
Flowering time (no. of days)	14,65	2.06	<b>0.026</b>	0.48	0.633

opposite was true for  $\delta^{13}\text{C}$  in both treatments, and for flowering time under water restriction (Table 4). For  $\delta^{13}\text{C}$  variance among lines was higher in allotetraploids than in diploids in both treatments (Table 4), as was flowering time under water restriction (Table 4). Among-population fraction of phenotypic variance was slightly higher for diploids than for allotetraploids in the well-watered treatment. Under water restriction, such fraction was higher for diploids than for allotetraploids for  $\delta^{13}\text{C}$  and flowering time but lower for carbon gain and  $g_s$  (Table 4).

**CLIMATIC CORRELATES**

We found significant associations between trait variation and climate only across allotetraploid populations. Relationships of  $g_s$  and  $\delta^{13}\text{C}$  with climatic factors were nonlinear (Fig. 3A–D). Pop-

ulations with low or high precipitation and soil moisture deficit had higher  $g_s$ , whereas intermediate climates had minimal stomatal conductance (Fig. 3A, B). A similar trend was also observed for photosynthesis and soil moisture deficit, although significance was marginal ( $P < 0.1$ , Fig. S8B).  $\delta^{13}\text{C}$  was maximum in populations with mid-values of precipitation and soil moisture deficit and decreased in populations with higher or lower aridity (Fig. 3C, D). Genotypic variation in photosynthesis correlated positively with temperature and negatively with precipitation, although significance was marginal ( $P < 0.1$ ; Fig. S8C, D). Flowering time correlated positively with precipitation although significance was also marginal ( $P < 0.1$ , Fig. S8E).

We found significant associations between trait plasticity and climate only across diploids populations. The relationship

**Table 3.** Coefficient of variation of physiological traits and flowering time in *Brachypodium distachyon* diploid (within each cell, data above = D) and *B. hybridum* allotetraploid (within each cell, data below = T) lines under two watering regimes. A summary of the results of the general or generalized linear mixed analyses conducted to test for broad-sense genetic variation within and between populations in these traits is also shown. Significant values ( $P < 0.05$ ) are in bold.

Well watered						
Source	Line (population)			Population		
Trait	Coefficient of variation (%)	Z	P	df	F	P
A ( $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ )	D: 69.19	0.02	0.983	13,87	5.19	<b>&lt;0.0001</b>
	T: 73.64	1.01	0.312	14,79	3.92	<b>&lt;0.0001</b>
$g_s$ ( $\text{mmol m}^{-2}\text{s}^{-1}$ )	D: 152.85	0.6	0.549	13,83	7.3	<b>&lt;0.0001</b>
	T: 131.50	0.24	0.814	14,77	6.19	<b>&lt;0.0001</b>
Integrated WUE ( $\delta^{13}\text{C } \text{‰}$ )	D: 3.34	0.07	0.946	13,28	8.97	<b>&lt;0.0001</b>
	T: 3.26	1.88	<b>0.029</b>	14,31	1.40	0.211
Flowering time (no. of days)	D: 48.29	5.0	<b>&lt;0.0001</b>	13,74	19.87	<b>&lt;0.0001</b>
	T: 54.66	4.21	<b>&lt;0.0001</b>	14,81	17.85	<b>&lt;0.0001</b>
Water restricted						
A ( $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ )	D: 107.6	1.11	0.268	13,75	2.27	<b>0.014</b>
	T: 98.6	0.87	0.384	14,66	4.34	<b>&lt;0.0001</b>
$g_s$ ( $\text{mmol m}^{-2}\text{s}^{-1}$ )	D: 125.79	0.31	0.758	13,72	3.55	<b>0.0003</b>
	T: 125.33	0.47	0.635	14,72	6.11	<b>&lt;0.0001</b>
Integrated WUE ( $\delta^{13}\text{C } \text{‰}$ )	D: 4.01	0.97	0.333	13,33	7.80	<b>&lt;0.0001</b>
	T: 4.34	1.74	<b>0.041</b>	14,34	2.25	<b>0.027</b>
Flowering time (no. of days)	D: 54.54	2.93	<b>0.0034</b>	12,40	5.53	<b>&lt;0.0001</b>
	T: 58.74	3.62	<b>0.0003</b>	13,48	2.94	<b>0.0032</b>

**Table 4.** Fraction of total phenotypic variance of physiological drought tolerance related traits (and flowering time) accounted for within and among populations in *Brachypodium distachyon* (diploid) and *B. hybridum* (allotetraploid) accessions sampled across the Iberian Peninsula and grown under two different watering treatments.

Well watered	Diploids			Allotetraploids			
	Traits	$V_{line}$	$V_{pop}$	$V_{res}$	$V_{line}$	$V_{pop}$	$V_{res}$
Well watered	A ( $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ )	0	<b>0.194</b>	<b>0.805</b>	0.085	<b>0.189</b>	<b>0.725</b>
	$g_s$ ( $\text{mmol m}^{-2}\text{s}^{-1}$ )	0.041	<b>0.328</b>	<b>0.631</b>	0.106	<b>0.281</b>	<b>0.642</b>
	Integrated WUE ( $\delta^{13}\text{C } \text{‰}$ )	0	<b>0.468</b>	<b>0.532</b>	<b>0.279</b>	0.086	<b>0.625</b>
	Flowering time (no. of days)	<b>0.177</b>	<b>0.704</b>	<b>0.118</b>	<b>0.164</b>	<b>0.603</b>	<b>0.231</b>
Water restricted							
Water restricted	A ( $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ )	0.107	<b>0.125</b>	<b>0.768</b>	0.091	<b>0.203</b>	<b>0.706</b>
	$g_s$ ( $\text{mmol m}^{-2}\text{s}^{-1}$ )	0.021	0.13*	<b>0.848</b>	0	<b>0.19</b>	<b>0.81</b>
	Integrated WUE ( $\delta^{13}\text{C } \text{‰}$ )	0	<b>0.347</b>	<b>0.653</b>	<b>0.187</b>	<b>0.143</b>	<b>0.67</b>
	Flowering time (no. of days)	<b>0.247</b>	<b>0.478</b>	<b>0.274</b>	<b>0.473</b>	<b>0.213</b>	<b>0.313</b>

Variance components are standardized to sum to 1. Variance components significantly different from zero ( $P < 0.05$ ) are in bold.

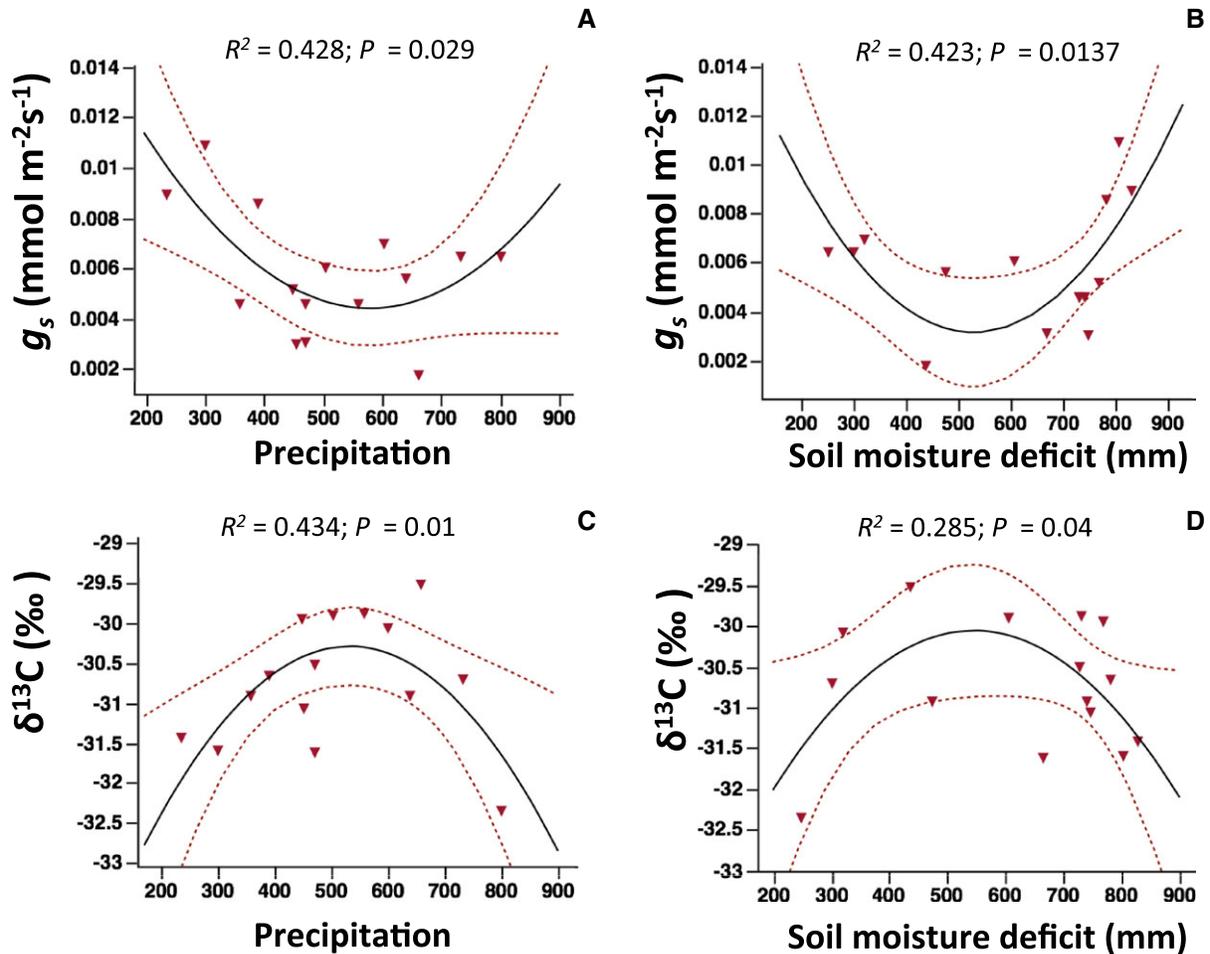
\* $P < 0.1$ .

between  $\delta^{13}\text{C}$  plasticity and soil moisture deficit was nonlinear (Fig. 4A).  $\delta^{13}\text{C}$  plasticity was maximum in populations with intermediate values of soil moisture deficit and decreased in populations with higher or lower aridity. Plasticity in flowering time increased with precipitation and decreased with soil moisture deficit (Fig. 4B, C). We detected no significant climatic rela-

tionships among climate and the remaining genetically variable traits (Table 2).

#### GENETIC CORRELATIONS

For diploids, all traits were significantly genetically correlated with each other in both treatments (Table 5). We found positive



**Figure 3.** Relationships between annual precipitation and annual soil moisture deficit and the genetic population means of ecophysiological traits under water-restricted conditions: (A, B) stomatal conductance ( $g_s$ ), (C, D) carbon isotope composition ( $\delta^{13}C$  ‰). Soil moisture deficit is defined as potential evapotranspiration minus precipitation. In all cases, the solid line depicts the best quadratic regression fit between variables, and dashed lines are its 95% confidence intervals. Only data for *Brachypodium hybridum* allotetraploids are depicted (red triangles).

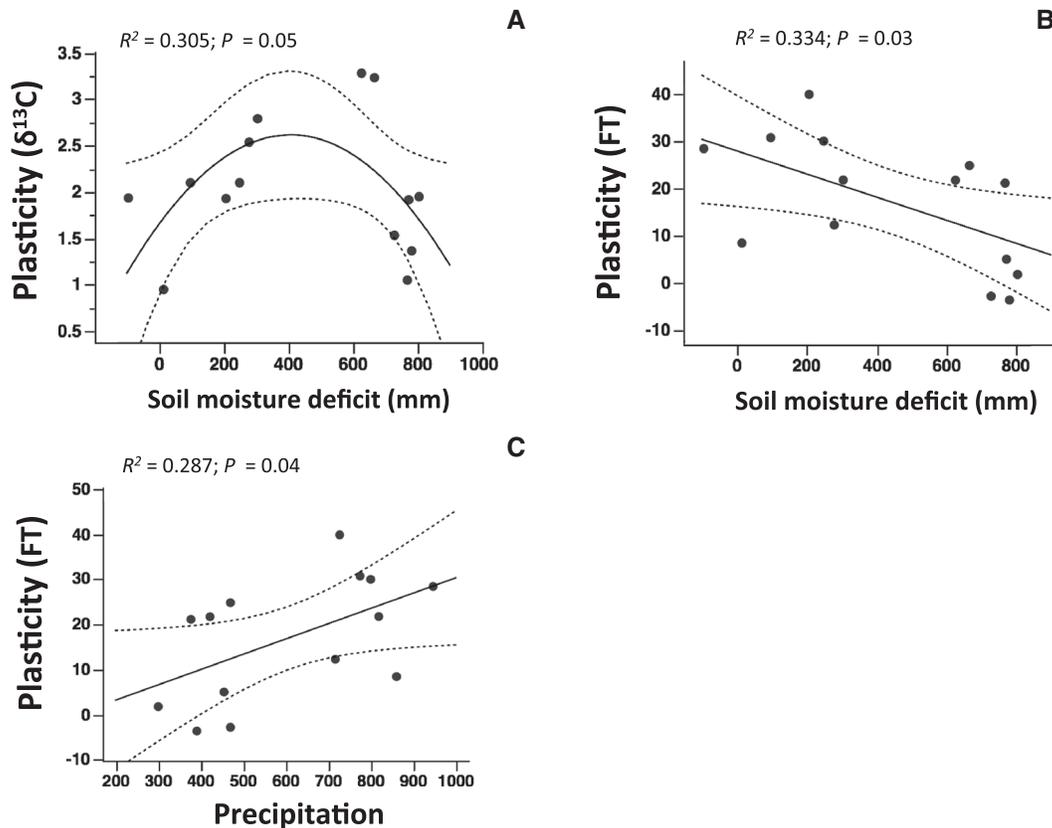
genetic correlations between photosynthesis and  $g_s$ , and between  $\delta^{13}C$  and flowering time in both treatments, and negative correlations for the remaining traits (Table 5). For allotetraploids, the number of significant genetic trait correlations was overall lower. We found a significant positive genetic correlation between photosynthesis and  $g_s$  under water restriction (Table 5). Flowering time was negatively correlated with photosynthesis and  $g_s$  in both treatments (Table 5). For both ploidy levels, the sign of genetic correlations never changed across treatments.

The analyses of random skewers showed that response vectors of G-matrices among ploidy lineages were not proportional under water restriction (vector correlation = 0.81,  $P = 0.948$ ), yet they were in the well-watered treatment (vector correlation = 0.85,  $P = 0.962$ ). In this last treatment, however, response vectors were larger in allotetraploids (response vector length ratio diploid/allotetraploid = 0.81), suggesting that the response to selection may be weaker in diploids. Response vectors dif-

fered between treatments in diploids (vector correlation = 0.80,  $P = 0.947$ ), but not in allotetraploids (vector correlation = 0.87,  $P = 0.968$ ). The response to selection of allotetraploids may be stronger under well-watered conditions (response vector length ratio water-restricted/well-watered = 0.72).

### Discussion

Until recently, the mechanistic basis of physiological variation underlying differential cytotype distribution was not well understood (but see Buggs and Pannell 2007; Hao et al. 2013). Here, we demonstrate that *Brachypodium* diploids and polyploids differ in gas exchange physiology and flowering time under water limitation. In dry conditions, allotetraploids maintain higher photosynthesis and stomatal conductance, and flower earlier, than diploids, concordant with a drought-escape strategy. For allotetraploids, ecophysiology varied as a function of precipitation and



**Figure 4.** Relationships among annual precipitation, annual soil moisture deficit, and population means of plasticity in ecophysiological traits: (A) carbon isotope composition, (B, C) flowering time (FT). Soil moisture deficit is defined as potential evapotranspiration minus precipitation. Plasticity values come from trait differences between the two watering treatments. In all cases, solid lines depict the best linear or quadratic regression fit between variables. Only data for *Brachypodium distachyon* diploids are depicted by green circles in the graphs.

aridity of the provenance populations. Furthermore, consistent with our expectations, adaptive evolution of ecophysiology is likely constrained by low levels of genetic variability and the presence of genetic correlations, in particular for *B. distachyon* diploid genotypes existing naturally in wet habitats. In addition, we found extensive genetic trait differentiation among Iberian *Brachypodium* populations. Consistent with predictions, standing within-population genetic variation in integrated WUE and flowering phenology was greater in allotetraploids than diploids. Overall, our study indicates that the divergence in ecological tolerance to water stress among *Brachypodium* ploidy lineages is likely an important driver of ecogeographical differentiation in this species complex (Manzaneda et al. 2012).

#### ADAPTIVE SIGNIFICANCE OF TRAIT DIFFERENTIATION AND PLASTICITY

Here, we show that trait differentiation among *Brachypodium* ploidies depends on environment for photosynthesis, flowering time, and to some extent, stomatal conductance, whereas differentiation in integrated WUE was essentially genetic. The elevated

photosynthetic performance of diploids under well-watered conditions suggests that *B. distachyon* diploids are better adapted than allotetraploids to humid environments. In contrast, allotetraploids maintain higher gas exchange rates, WUE, and accelerated flowering times in dry conditions, suggesting that *B. hybridum* allotetraploids might be better adapted—through a drought-escape strategy—than diploids to dry environments. Indeed, *B. distachyon* diploids are mainly found in humid locations, whereas *B. hybridum* allotetraploids occupy drier sites (Manzaneda et al. 2012). Although decreased WUE is also expected under water stress for short-lived annuals with a drought-escape strategy (as result of increasing stomatal conductance to increase carbon gain; e.g., McKay et al. 2003; Heschel and Riginos 2005), increased WUE could be advantageous in water-limited environments if it reduces development time and enhances the likelihood of survival to reproduction in short growing period (Wu et al. 2010). Indeed, under water stress, allotetraploids not only flowered significantly earlier than diploids, but they had about 65% higher probability of flowering. We found negative genetic correlations between flowering time and physiological traits for allotetraploids

**Table 5.** Genetic correlations ± SE among four ecophysiological traits for *Brachypodium distachyon* diploids (above the diagonal, WR: *N* = 90 lines, *N* = 47 lines for δ<sup>13</sup>C correlations; WW: *N* = 89 genotypes, *N* = 47 genotypes for δ<sup>13</sup>C correlations), and *B. hybridum* allotetraploids (below the diagonal, WR: *N* = 98 lines, *N* = 47 lines for δ<sup>13</sup>C correlations; WW: *N* = 94 genotypes, *N* = 46 genotypes for δ<sup>13</sup>C correlations) in the two water treatments.

WR	A	<i>g<sub>s</sub></i>	δ <sup>13</sup> C	Flowering time
A		<b>1.0 ± 0.023</b> <b><i>P</i> &lt; 0.0001</b>	<b>0.999 ± 0.21</b> <b><i>P</i> = 0.0003</b>	<b>-0.72 ± 0.10</b> <b><i>P</i> &lt; 0.0001</b>
<i>g<sub>s</sub></i>	<b>0.95 ± 0.072</b> <b><i>P</i> = 0.0001</b>		–	<b>-0.79 ± 0.103</b> <b><i>P</i> &lt; 0.0001</b>
δ <sup>13</sup> C	0.60 ± 0.34 <i>P</i> = 0.107	0.62 ± 0.65 <i>P</i> = 0.32		<b>-0.83 ± 0.13</b> <b><i>P</i> &lt; 0.0001</b>
Flowering time	<b>-0.89 ± 0.10</b> <b><i>P</i> &lt; 0.0001</b>	<b>-0.86 ± 0.12</b> <b><i>P</i> &lt; 0.0001</b>	-0.53 ± 0.19 <i>P</i> = 0.0121	
WW	A	<i>g<sub>s</sub></i>	δ <sup>13</sup> C	Flowering time
A		<b>0.98 ± 0.043</b> <b><i>P</i> &lt; 0.0001</b>	<b>0.86 ± 0.14</b> <b><i>P</i> &lt; 0.0001</b>	<b>-0.81 ± 0.097</b> <b><i>P</i> &lt; 0.0001</b>
<i>g<sub>s</sub></i>	–		<b>0.62 ± 0.15</b> <b><i>P</i> = 0.0013</b>	<b>-0.66 ± 0.094</b> <b><i>P</i> &lt; 0.0001</b>
δ <sup>13</sup> C	0.20 ± 0.31 <i>P</i> = 0.53	0.45 ± 0.74 <i>P</i> = 0.53		<b>-0.74 ± 0.11</b> <b><i>P</i> &lt; 0.0001</b>
Flowering time	<b>-0.71 ± 0.105</b> <b><i>P</i> &lt; 0.0001</b>	<b>-0.80 ± 0.14</b> <b><i>P</i> &lt; 0.0001</b>	-0.35 ± 0.21 <i>P</i> = 0.12	

Genetic correlations were estimated from variance components in REML (Proc Mixed) using individual level data. Traits were standardized to a mean of 0 and SD of 1. Uncorrected *P*-values are presented beneath the genetic correlations. Significant genetic correlations after Bonferroni's correction for six tests for each ploidy level ( $\alpha = 0.05/6 = 0.008$ ) are in bold.

(–), nonestimable because model fails to converge.

WW, well watered; WR, water restricted.

under water stress, indicating that genetic lines with higher photosynthesis and stomatal conductance flower early (Fig. S9). Negative, genetically-based correlations between gas exchange physiology and flowering time have been reported previously in annuals species and have been interpreted as evidence of drought escape (e.g., Geber and Dawson 1997; McKay et al. 2003; Heschel and Riginos 2005; Sherrard and Maherali 2006; Donovan et al. 2007; Wu et al. 2010).

Allotetraploids maintained higher integrated WUE in dry conditions than diploids, likely owing to a significant reduction in stomatal conductance, but no change in photosynthesis under water stress. Therefore, ecophysiological variation among ploidies under water restriction may reflect cytotypic differences in stomatal anatomy and conductance, as well as differences in photosynthetic capacity (Geber and Dawson 1997; McKay et al. 2003). Indeed, whole-genome duplications can have large effects on plant anatomy and gas exchange biochemistry (Warner and Edwards 1993; te Beest et al. 2012). In the case of allopolyploids, however, characteristics of naturally found polyploid plants represent not only the inherent effects of increased chromosome number, but also past interspecific hybridization and the results of natural selection after polyploidization (Otto 2007; Maherali et al. 2009). *Brachypodium hybridum* allotetraploids have a polytypic origin resulted from ancient bidirectional crosses of *B.*

*distachyon* and *B. stacei* plants acting either as maternal or paternal parents (López-Alvarez et al. 2012). Environmental niche data have shown that *B. distachyon* is found in higher, cooler, and wetter places than *B. stacei*, which grows in lower, warmer, and drier environments (López-Alvarez et al. 2015). Although *B. hybridum* may also be found in zones with intermediate climates, it frequently grows in low altitudinal warmer and drier places, like its *B. stacei* progenitor (Manzaneda et al. 2012; López-Alvarez et al. 2012, 2015). In fact, across its native range of distribution sympatric admixed populations of *B. stacei* and *B. hybridum* have been found in several localities (López-Alvarez et al. 2015; A. J. Manzaneda, pers. obs.). Interestingly, both species share common life-history traits as rapid plant development, lack of vernalization, and early flowering (Iberian *B. stacei* lines bloom about 31–39 days after germination; Martínez and Manzaneda, unpubl. ms.), which are typical features associated to drought escape. Our results contrast with the only other study of adaptive ecophysiological differentiation in heteroploid annual species, which found that diploid *Mercurialis annua* are more drought tolerant than polyploids (Buggs and Pannell 2007). Polyploid *Mercurialis annua* showed lower WUE and photosynthesis and higher transpiration than diploids under water limitation (Buggs and Pannell 2007). Physiological responses to water stress of cytotypes are likely species specific or could occur via evolutionary changes

after polyploidization (Maherali et al. 2009). More studies of heteroploid annuals are needed to evaluate what functional strategy drives adaptive differentiation of polyploids in drier environments.

Fitness and trait data from field studies are necessary to demonstrate whether WUE and gas exchange traits are adaptive. However, adaptive differentiation of ecophysiological traits may also be inferred from the analysis of clinal variation across environmental gradients (e.g., Maron et al. 2007; Paccard et al. 2014). Our correlations between physiology and climate provide additional evidence for adaptive trait differentiation of allotetraploids. First, we detected a significant correspondence between physiological trait variation and climate only across allotetraploid populations. Consistent with a drought-escape strategy, allotetraploid lines from warmer and/or drier populations tended, overall, to have higher photosynthesis and earlier flowering than lines from cooler and/or wetter populations (Fig. S8). However, the strongest relationships between plant functional traits and aridity were not linear. Thus, stomatal conductance and photosynthesis were lowest at intermediate climates and enhanced at both extremes of the aridity gradient. Likewise, WUE peaked in lines from intermediate levels of aridity or annual precipitation. In  $C_3$  plants WUE is expected to increase with aridity (Prentice et al. 2011; Cernusak et al. 2013). The nonlinear relationships likely reflect the nonlinear soil water availability across allotetraploids populations (Fig. S5). When aridity is on the low-to-moderate sides of the gradient, increasing aridity is correlated with reduced stomatal conductance and photosynthesis and enhanced WUE, characteristic of a typical dehydration-avoidance response. However, at the driest end of the aridity gradient where selection pressure exerted by water stress is presumably strongest, increasing aridity corresponds with increased stomatal conductance and photosynthesis and diminished WUE, consistent with a drought-escape strategy. Adoption of mixed strategies of resistance and escape in response to drought has been documented previously (e.g., Paccard et al. 2014). Our results suggest mixed strategies across *B. hybridum* populations that could emerge as the result of a differential adaptation to nonlinear variations in water availability across the species range. Clinal differentiation among populations in ecophysiology indicates that *B. hybridum* allotetraploids may have adapted to differences in aridity along climatic gradients, likely resulting from divergent selection exerted by water availability (Ackerly et al. 2000; Donovan et al. 2009). In contrast, trait differentiation among diploid populations may not be adaptive, as we found no relationship between trait variation and climate in diploids. Population differentiation in diploids could be caused by historical or demographical factors, and maintained by limited gene flow between populations (*B. distachyon* is strongly selfing; Vogel et al. 2009). Reciprocal transplant studies will be required to test whether population differentiation is adaptive.

Many plant species show plasticity in ecophysiological traits when exposed to environments with contrasting water availability (e.g., Agrawal et al. 2008; Ivey and Carr 2012; reviewed in Nicotra and Davidson 2010). We predicted that polyploids would maintain greater plasticity than diploids because of higher levels of heterozygosity and novel genetic and genomic rearrangements derived from polyploidization (te Beest et al. 2012; Hahn et al. 2012). However, we did not detect significantly higher trait plasticity in allotetraploids compared to diploids. In contrast, plasticity in photosynthesis was higher in diploids than allotetraploids. Few studies have compared plasticity among related ploidy lineages, and overall patterns remain ambiguous and trait dependent (e.g., Bretagnolle and Thompson 2001; Hahn et al. 2012; te Beest et al. 2012). Only for *B. distachyon* diploids was plasticity related to climate. For diploids, plasticity in WUE varied nonlinearly with aridity. The highest values of WUE plasticity were found for populations with intermediate levels of aridity. Plasticity in flowering time varied linearly with aridity/precipitation; higher plasticity in flowering time was found for humid populations. If plasticity in WUE and flowering time is adaptive at mild and wet climates, it could have facilitated cytotypic differentiation and establishment of *B. distachyon* diploids in such environments.

#### LIMITATIONS ON ADAPTIVE EVOLUTION OF DROUGHT-RESPONSE TRAITS IN *BRACHYPODIUM*

In our study, ecophysiological traits varied among *Brachypodium* lines and populations. Previous studies have documented significant natural variation in leaf water content and photochemical efficiency (Luo et al. 2011), flowering and vernalization time (Schwartz et al. 2010), and root system architecture (Pacheco-Villalobos and Hardtke 2012) among *B. distachyon* accessions from Turkey and Iraq. Those results, and ours from Iberian populations, demonstrate extensive natural variation in drought-response traits across the native range of *B. distachyon*. Yet, little is known about the adaptive significance of this variation (Pacheco-Villalobos and Hardtke 2012; IBI 2014).

We predicted that allotetraploids would have more genetic variation in ecophysiology than diploids because of increased heterozygosity. As expected, we detected greater genetic variation within populations of allotetraploids than diploids in  $\delta^{13}C$  in both experimental conditions, and in flowering time under water limitation. Both allotetraploids and diploids maintained low levels of standing genetic variation in the remaining traits, which could limit their adaptive evolution (Caruso et al. 2005). We predict that response to natural selection on  $\delta^{13}C$  and flowering time should be faster in allotetraploid than in diploid lines, although conclusions about the limits of adaptive evolution in diploids should be taken with caution given the relative low number of lines per population included in this study. Other studies have also reported low heritability of ecophysiological traits associated with drought

response (e.g., Heschel et al. 2002; Sherrard and Maherali 2006; Donovan et al. 2007, 2009), and may reflect strong directional selection in the past (Ackerly et al. 2000; see also Geber and Griffen 2003), or the existence of a strong environmental component to measuring gas exchange, which might also reduce the ability to detect additive genetic variation (e.g., Ackerly et al. 2000).

Empirical studies comparing response to selection among ploidy lineages are scarce and it is still unclear whether genome duplication typically increases or decreases the species' ability to respond to selection (see Martin and Husband 2012). However, our results are consistent with studies of *Brassica napus* that have documented extensive de novo variation in life-history traits, including flowering time, in new-formed allopolyploid lines compared with their diploid parents (Schranz and Osborn 2004). For both ploidies, levels of genetic variation in ecophysiological traits were significantly higher among populations than within populations indicating substantial population differentiation in physiology. In well-watered conditions, with the exception of  $\delta^{13}\text{C}$ , population-level phenotypic variance was only slightly higher in diploids than allotetraploids. In dry conditions, this pattern was more unpredictable and trait dependent, but for  $\delta^{13}\text{C}$ , the amount of phenotypic variance among populations was clearly higher (about 36.6%) in diploids than in allotetraploids.

Consistent with our predictions, we found that allotetraploids show weaker genetic correlations than diploids. For diploids, all traits were significantly correlated with each other in both treatments, whereas for allotetraploids the number of significant genetic correlations was lower. Likewise, genetic covariances tend to constrain the responses to selection vectors in similar directions but to varying degrees in both ploidy lineages. Under water restriction, G-matrices were not entirely proportional among ploidy lineages suggesting different trajectories in the evolution of ecophysiological traits in dry conditions. In wet conditions, although G-matrices were proportional among ploidy lineages, the response vector differed in magnitude suggesting a weaker response to selection of diploids, which is likely caused by a stronger trait correlational structure. As the evolutionary response to natural selection may be constrained by the genetic-covariance matrix, changes in G-matrices associated with polyploidy and/or ancient hybridization could allow allotetraploids to diversify rapidly even if diploid and allotetraploids experience similar patterns of selection (Oswald and Nuismer 2010).

Diploids are primarily found in mesic populations, so we hypothesize that evolution of ecophysiological traits should be constrained across diploid lines and populations because of the existence of strong genetic correlations in humid conditions, consistent with the lack of clinal trait differentiation observed across diploid populations. We are not aware of any study comparing G-matrices of diploid and allopolyploid lineages, but these re-

sults are expected from the fact that epigenetic changes, changes in gene expression and chromosomal rearrangements, are typically observed in allopolyploids (Oswald and Nuismer 2010; Martin and Husband 2012) that may underlie the weaker genetic correlations observed for *B. hybridum* allotetraploids. Similarly, proportionality or magnitude of G-matrices differed between watering treatments. For diploids, response vectors of G-matrices were not proportional between watering treatments. For allotetraploids, response vectors differed in magnitude, suggesting that the response to selection may be stronger in wet conditions. Our results are concordant with other studies that reported variations in physiological G-matrices across watering environments (e.g., Sherrard et al. 2009).

Finally, we found pairwise negative genetic correlations among gas exchange physiology,  $\delta^{13}\text{C}$ , and flowering time, which could indicate pleiotropy, and might reflect trade-offs among different drought strategies (e.g., McKay et al. 2003; Heschel and Riginos 2005; Sherrard and Maherali 2006; Donovan et al. 2007). As expected, we found positive genetic correlations among gas exchange traits, which likely reflect trade-offs between carbon fixation and water loss through the stomata (Geber and Dawson 1997). Both negative and positive genetic correlations could hinder adaptation if the direction of selection is the same for negatively correlated traits, or different for positively correlated traits (Etterson and Shaw 2001; Caruso et al. 2005). Whether such trait genetic correlations are in the same direction as selection (e.g., correlational selection) or opposed to selection still would require experimental verification in natural populations of both ploidy lineages.

## Conclusion

Our results support the hypothesis that differences in tolerance to water stress may underlie ecological divergence of closely related ploidy lineages growing under contrasting environments (Ramsey 2011; Martin and Husband 2013). Adaptive evolution of ecophysiological traits could be constrained by the absence of sufficient trait genetic variation and the structure of genetic correlations among traits. Standing genetic variation and clinal variation of traits differed between ploidies and water environments, suggesting that response to selection of some traits, and their evolutionary trajectories, may be different for each ploidy lineage.

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## DATA ARCHIVING

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Figure S1.** Map of the Iberian Peninsula showing the location of the *Brachypodium* populations included in the study.

**Figure S2.** Soil moisture variation during the drought-tolerance experiment between the two watering treatments.

**Figure S3.** A picture of the experiment taken at the seventh week within the Duke Green house.

**Figure S4.** LI-COR LI-6400 portable photosynthesis system modified to fit a set of four whole-plant cuvettes in a parallel processing system.

**Figure S5.** Nonlinear relationships among annual soil moisture deficit, latitude, and longitude across *Brachypodium distachyon* diploid populations (graphs above, green circles), and *B. hybridum* allotetraploids (graphs below, red triangles).

**Figure S6.** Interindividual natural variation in whole-plant photosynthesis.

**Figure S7.** Interindividual natural variation in whole-plant photosynthesis.

**Figure S8.** Relationships among annual precipitation, temperature, and annual soil moisture deficit and the genetic population means of ecophysiological traits under water-restricted conditions.

**Figure S9.** Genetic relationships across ecophysiological traits.

**Table S1.** Localities and geographical coordinates of the 24 *Brachypodium distachyon* (diploids) and *B. hybridum* (allotetraploids) populations across the Iberian Peninsula used in this study.

**Table S2.** Location of 90 *Brachypodium distachyon* (diploids) and 99 *B. hybridum* (allotetraploids) accessions included in the drought experiment conducted to characterize natural variation in ecophysiological traits.

**Table S3.** Ecophysiological traits and flowering-time mean differences (plasticity) between two contrasting watering treatments for *Brachypodium distachyon* diploids and *B. hybridum* allotetraploids.

**Table S4.** Significance of the covariate “number of spikes” (i.e., the number of spikes produced at the time of the gas exchange measurement) on the variation in whole-plant gas exchange among diploid and allotetraploid lines under well-watered and water-restricted conditions.