Adaptive differentiation among populations of the Mediterranean dry grassland species *Brachypodium retusum*: The role of soil conditions, grazing, and humidity

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PREMISE OF THE STUDY: Genetic differentiation in plant species may result from adaptation to environmental conditions, but also from stochastic processes. The drivers selecting for local adaptation and the contribution of adaptation to genetic differentiation are often unknown. Restoration and succession studies have revealed different colonization patterns for *Brachypodium retusum*, a common Mediterranean grass. In order to understand these patterns, we tested population differentiation and adaptation to different environmental factors.

METHODS: Structured sampling of 12 populations from six sites and two soil types within site was used to analyze the spatial and environmental structure of population differentiation. Sampling sites differ in grazing intensity and climate. We tested germination and growth in a common garden. In subsets, we analyzed the differential response to stone cover, grazing and soil moisture.

KEY RESULTS: We found significant differences among populations. The site explained population differentiation better than soil, suggesting a dominant influence of climate and/or genetic drift. Stone cover had a positive influence on seedling establishment, and populations showed a differential response. However, this response was not related to environmental differences between collection sites. Regrowth after clipping was higher in populations from the more intensively grazed Red Mediterranean soils suggesting an adaptation to grazing. Final germination was generally high even under drought, but germination response to differences in soil moisture was similar across populations.

CONCLUSIONS: Adaptive population differentiation in germination and early growth may have contributed to different colonization patterns. Thus, the provenance of *B. retusum* needs to be carefully considered in ecological restoration.

KEY WORDS Common garden; local adaptation; plant origin; Poaceae; population differentiation; seedling recruitment.

Genetic differentiation among populations is an important strategy of plant species to survive under different environmental conditions (Linhart and Grant, 1996; Bischoff and Hurault, 2013). Adaptive differentiation occurs if environmental gradients are strong and gene flow counteracting differentiation is low (Kawecki and Ebert, 2004). Climate is a major driver of local adaptation and genetic differentiation in plant populations, in particular on large geographic scales (Etterson, 2004; Leinonen et al., 2009; Bucharova et al., 2016). However, microclimatic gradients may also act on much smaller scales resulting in small-scale adaptation and differentiation (Knight and Miller, 2004; Lenssen et al., 2004; Bischoff et al., 2006).

The adaptation to soil conditions was first detected on soils contaminated with heavy metals indicating that adaptive differentiation may occur quite rapidly (Schat et al., 1996; Antonovics, 2006). Furthermore, the pH value and related chemical and biotic properties may represent a strong selective force (Macel et al., 2007; Raabová et al., 2011). Humidity is also a limiting factor for plant growth and reproduction leading to drought adaptation (Shah and Paulsen, 2003; Suriyagoda et al., 2010). However, an increase in soil

moisture often results in higher competition for other resources that represent a counteracting selective force (Kadmon, 1995).

Ecosystem management is a third potential driver of adaptive differentiation in semi-natural systems such as grasslands. Grazing changes site conditions by biomass removal, plant damage, reduced competition, and creates heterogeneity in grassland vegetation (Bullock et al., 2001; Hufford and Mazer, 2012). It seems to be likely that long-term grazing favors genotypes adapted to grazing (Hufford and Mazer, 2012). However, grazing may also obscure adaptation to other environmental factors if this selection pressure has not been consistent in the past (Hufford et al., 2008).

Genetic differentiation is not only the result of adaptation to different environments. Stochastic processes such as genetic drift considerably contribute to population differentiation and may even overwhelm local adaptation (Hereford and Winn, 2008; Hereford, 2009). Genetic drift and limited dispersal favor isolation by distance effects that are not related to environmental gradients (Sexton et al., 2013). Durka et al. (2017) found both isolation by distance caused by stochastic processes, and isolation by environment in seven tested plant species. The contribution of adaptive differentiation to genetic structure may even be low compared with genetic drift (Michalski and Durka, 2012, Sexton et al. 2013).

Genetic differentiation in quantitative, and thus potentially adaptive traits, is typically tested in common garden experiments (Petit et al., 2001; Leinonen et al., 2009; Bischoff and Müller-Schärer, 2010). Tests for local adaptation require reciprocal transplantation to original collection sites (Blanquart et al., 2013). However, common garden experiments comparing the response to the manipulation of key environmental factors allow conclusions on adaptive differentiation and may reveal more specific information on drivers of local adaptation than reciprocal transplantations in the field (Lenssen et al., 2004; Pahl et al., 2013).

We used the perennial grass *Brachypodium retusum* (Pers.) P. Beauv**.** as a model species to test for neutral and adaptive population differentiation at a regional scale in Southern France. The species is dominant in dry grasslands of the Western Mediterranean basin, but also occurs in matorral and open woodlands (Caturla et al., 2000; Catalan et al., 2015). In contrast to studies showing a high colonization capacity of the species (Caturla et al., 2000; Luis et al., 2004; Cassagne et al., 2011), spontaneous natural regeneration of *B. retusum* was poor in the La Crau nature reserve (Southern France) 30 years after conversion from arable use to traditional sheep grazing, complicating ecological restoration operations (Coiffait-Gombault et al., 2012). We hypothesize that genetic differentiation contributes to the different colonization patterns, and that this differentiation results from adaptation to major environmental factors distinguishing habitats. In the study region, *B. retusum* occurs in lower calcareous mountain ranges and on more or less decalcified red Mediterranean soils of former riverbeds such as the Crau area. The latter habitat is also characterized by stones (pebbles and cobbles) shaped by the former rivers. These stones were found to favor growth and survival of *B. retusum* because rhizomes and roots can grow underneath and thus benefit from higher soil moisture during the hot and dry Mediterranean summer (Buisson et al., 2015). Apart from soil characteristics and stone cover, these two habitats show also large differences in soil moisture and grazing intensity. The information on population differentiation and adaptation to environmental factors is crucial to predict colonization processes, but also to improve seed sourcing in ecological restoration (McKay et al., 2005; Vander Mijnsbrugge et al., 2010).

In our study, we tested *B. retusum* populations from calcareous and closest red Mediterranean soils replicated at different geographical distances to evaluate whether local-scale differentiation along strong environmental gradients can be as important as large-scale differentiation driven by macroclimate or genetic drift (Bischoff et al., 2006; Hereford and Winn, 2008; Durka et al., 2017). We further analyzed differential responses of these populations to grazing simulated by clipping, to stone cover and to soil moisture. We specifically wanted to answer the following research questions:

(1) At which scales does population differentiation occur: what is the role of local-scale soil type compared with large-scale variation? (2) Is this differentiation adaptive in terms of soil conditions (stone cover), management (grazing simulated by clipping) and humidity (soil moisture, germination)?

MATERIAL AND METHODS

Study species

Ramose false brome (Poaceae, *Brachypodium retusum* P. Beauv.) is a rhizomatous perennial C3 grass species. Its rhizomes form a dense network under the soil surface, resulting in a high resilience to aboveground disturbance such as wildfires or grazing (Caturla et al., 2000). Vegetative growth is characterized by tillering at the stem base, but also by lateral branching at upper stem parts. *Brachypodium retusum* is an outcrossing, wind-pollinated species. It flowers from April to July and shows high clonal growth and reproduction when rhizome connections disintegrate. However, rhizomes are short and just allow a slow clonal spread (< 5 cm × year^{-1} in our experiments).

Collection sites and sampling

The study and sampling sites were situated in the central part of the French Mediterranean climate zone from Montpellier to the lower Durance valley. Seeds were collected from six populations on base-rich calcareous soils (Calcaric cambisol) of lower mountain ranges with pH values of 7.8–8.9 (Table 1, Fig. 1). Close to each of these six populations on calcareous grasslands, six populations were also collected from usually decalcified red Mediterranean soils (Haplic cambisols) with pH values of 6.7–8.0. Three additional populations were collected in the Crau nature reserve as the focal site of Mediterranean dry grassland restoration (Buisson and Dutoit, 2006) in order to obtain a better representation of this largest red Mediterranean soil area of the study region. For further analysis, the four Crau populations (distance: 4–11 km) were pooled to the red Mediterranean soil population of Saint-Martin-de-Crau (SM, Table 1).

Red Mediterranean soils show a higher clay content than calcareous soils, resulting in a better nutrient and water retention. Because of the higher soil fertility, grasslands on red Mediterranean soils were more intensively used (in particular grazing, less frequently mowing) than those on calcareous soils (Table 1). They are also rich in hydrated oxides of iron and aluminum, and in silica whereas calcareous soils are poorer in these elements. Up to 50% of red Mediterranean soil surface is covered by cobbles and pebbles deposited by former rivers. Calcareous soils are less deep on calcareous bedrock and contain numerous angular brittle stones.

The study area is characterized by a typical Mediterranean climate with hot and dry summers, and mild and humid winters. The

TABLE 1. Soil characteristics, grazing intensity, annual precipitation, mean annual air temperature (including January and July mean in brackets), and the number of frost days (T<0) at collection sites of the twelve populations. Sites are ordered from east to west. Climate data are based on daily averages from the nearest meteorological stations (1980-2010). *, Irregular mowing.

Site code	Site name	Soil	Coordinates	pH	Grazing	Precipitation (mm*a^{-1})	Temperature (°C) Mean [Jan/Jul]	Days T<0
Me	Mérindol	Calcareous	43.756456 N, 5.199526 E	8.0	No*	583	14.5 [6.0/23.7]	49
		Red Mediterranean	43.750425 N, 5.210495 E	8.0	Low			
Ca	Caumont	Calcareous	43.898827 N, 4.941070 E	8.2	No	676	14.6 [5.9/24.2]	33
		Red Mediterranean	43.898319 N, 4.932395 E	6.9	No			
SR	Saint-Rémy-de-Provence	Calcareous	43.768047 N, 4.831898 E	7.8	No	625	15.1 [6.6/24.3]	30
		Red Mediterranean	43.798952 N, 4.841659 E	6.8	High			
SM	Saint-Martin-de Crau	Calcareous	43.705354 N, 4.797561 E	8.1	No	517	15.3 [7.4/24.4]	24
		Red Mediterranean	43.567465 N, 4.834645 E	6.8	High			
Ni	Nîmes	Calcareous	43.784700 N, 4.259389 E	8.0	No	701	14.9 [6.7/24.1]	34
		Red Mediterranean	43.722868 N, 4.339264 E	7.8	No*			
Mon	Montpellier	Calcareous	43.645416 N, 3.691638 E	8.9	No*	870	14.1[6.4/23.2]	51
		Red Mediterranean	43.607580 N, 3.449269 E	6.7	No*			

six sampling sites differ in climatic conditions (Table 1) whereas differences between soil types within sites are small. Annual precipitation depends on the proximity to mountain ranges and is generally higher in the western than in the eastern parts of the study area. Close to the Mediterranean Sea, winter temperatures are higher resulting in a lower frost frequency.

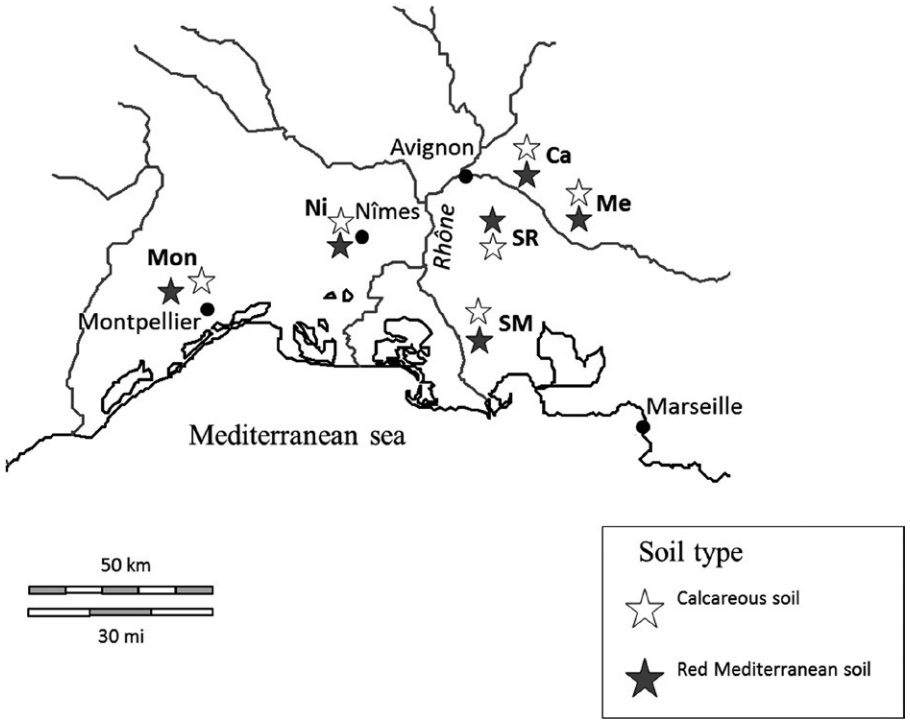


FIGURE 1. Position of six sampling sites each comprising two populations from different soil types.

Seed collection and preparation

In each population, all inflorescences of each of 50 1-m² patches were collected. The minimum distance between patches was 10 m and sampling was done in one or several parallel lines depending on population shape (linear versus polygon). Because of clonal growth of the species, the real patch size of individuals is unknown. A distance of 10 m was considered sufficient to obtain samples from different individuals (Montalvo and Ellstrand, 2000). On average, one patch comprised 40 inflorescences resulting in a total of 2000 collected inflorescences per population. In germination experiments, the number of seed families may be higher than the number of patches because one patch may comprise more than one individual. This pooling at patch level reduces intrapopulation variation compared to half-sib designs based on individual seed families. In all other experiments, only one seedling per patch was kept for planting, corresponding to classical half-sib designs.

Population differentiation under common conditions

Population differentiation was tested in a growth chamber germination experiment and in a common garden study. Inflorescences were shaken in tubes with glass beads using a vortex mixer to separate seeds from glumes. Transparency under transmitted overhead projector light was used as a criterion to remove empty seeds without embryos. In December 2016, seeds were germinated in peat plugs (4 cm × 4 cm). Four seeds of each sampled patch were sown per plug, and altogether, 40 patches of each population were randomly selected for a total of 160 seeds and 40 plugs per population. The plug trays were placed in a growth chamber (temperature 15/20°C, 12/12 h, night/day) and watered regularly. Coleoptile emergence used as a proxy of germination was recorded every 2–3 days for seven weeks. In March 2016, 24 plugs per population (corresponding to 24 patches or seed families) were randomly selected and transplanted to a previously ploughed experimental site in Avignon-Montfavet. Thus, 48% of the initially collected patches were represented in the field. Seedling number was reduced to one (average size) per plug prior to transplantation. Two plugs per population were planted to random positions within each of 12 blocks (3 m × 3 m each). During the first four months, seedlings were moderately watered. Plots were weeded by hand to limit competition.

Plant height, diameter, tiller number, leaf number, and the length of the longest leaf were measured once a month. Young *B. retusum* plants allocate resources to tillering and leaf growth. Later, tillers elongate and older leaves die. Therefore, we calculated cumulative leaf length as an aboveground biomass estimate for young seedlings (leaf length × number of leaves) and cumulative tiller length for older plants (plant height × number of tillers).

Adaptation to differences in soil conditions, management, and soil moisture

In May 2016, stones of 10–20 cm length and 7–15 cm width were placed in a circle around half of the seedlings of the common garden experiment to test their effect on plant growth. The stones were taken from the red Mediterranean soil of the Caumont population (Ca, Table 1, Fig. 1). Measurements were the same as in the population differentiation study under common conditions detailed above.

A subset of four population pairs (red Mediterranean and calcareous soils × Me, Ca, SR, SM sites) was grown one year earlier in a similar common garden experiment to simulate grazing by clipping established plants. Clipping of second year plants prevents high mortality compared to clipping of young seedlings. The design was similar to the differentiation study. In November 2014, seeds were sown in peat plugs and placed in a greenhouse. In February 2015, two seedlings of each population were planted in each of 12 blocks (24 seedlings in total). Because of low germination, seedling number was only one per block for the Me population from calcareous soil (12 seedlings in total). Plants were moderately watered during the first year, but not in the second year. In April 2016, half of the seedlings were clipped 2 cm above the soil. We measured the same traits as in the differentiation study. Additionally, the number of inflorescences per plant was counted at the end of the season. This was not possible in the population differentiation study under common conditions because plants rarely (<10%) flower in the first year.

The response to soil moisture during germination was analyzed in a controlled experiment manipulating moisture levels. The major objective was to test adaptive differentiation in germination response under dry conditions. Five moisture levels from 0.3 to 20% were adjusted by adding water to plastic boxes filled with 1.15 kg of sand and using a Theta Probe ML2x sensor (Delta-T Devices Ltd, Cambridge, United Kingdom). A subset of 10 populations was chosen for this experiment. Six seeds of each population (four for calcareous population of SM due to limited seed number) were sown in each of 12 boxes and each of the five soil moisture levels; this resulted in a total of 360 seeds per population assigned to 60 boxes. Boxes were closed with a translucent lid to keep initial moisture levels. The boxes were placed in a growth chamber with a 12-h cycle of light and temperature (day: 25°C; night: 12°C). Germination and moisture were measured every three days for a period of six weeks. Germinated seeds were removed. At the end of the experiment, the water potential of the sand was measured using WP4C Dewpoint Water Potentiometer (Decagon Devices, Pullman, Washington, USA). Water potentials were close to 0 (saturation) for the highest and –0.18 MPa for the lowest moisture level (Appendix S1, see Supplemental Data with this article).

Statistical analysis

Linear models were used to analyze population and treatment effects on germination and growth traits. Population was divided into a soil and site component. The two adjacent populations from red Mediterranean and calcareous soils were considered as originating from the same site resulting in a total of six sites and two soil types per site. Thus, each population represented a particular combination of site and soil. Site, soil, and their interaction were included as fixed effects in the models. The effect size of site of origin, soil of origin, and the site × soil interaction was calculated as 100 × factor sum of square/total sum of squares.

The common garden studies were designed as completely randomized block experiments; thus, block was included as an additional random factor. The adaptation treatments (stone cover, clipping) were included as a fixed factor for measurements taken after treatment start. In some cases $\log10(x + 1)$ transformation was required to comply with assumptions of linear models (normality, homoscedasticity).

In the germination experiments, final germination percentage in plugs (population differentiation study) and in boxes (soil moisture test) was calculated as response variable. Germination percentage was arcsin (sqrt(x/100)) transformed prior to analysis (Quinn and Keough, 2002). Because the position of plugs was completely randomized before transplantation to the field, no block effect was considered (germination in the population differentiation study). The soil moisture test on germination was analyzed in a split-plot model. The box effect was included as a random factor nested in moisture level. Accordingly, moisture was tested against the box effect whereas soil, site, and all interactions were tested against the model error.

A Tukey honest significance test (HSD) posthoc test was calculated to analyze differences between treatment levels more in detail if the treatment main effect or interactions were significant. Posthoc tests were preferred over a priori linear contrasts because we had no clear a priori hypothesis on specific adaptations to tested environmental factors. All statistical analyses were run in R (R, version 3.3.1, R Development Core Team (2013)).

RESULTS

Population differentiation under common conditions

A high variation in germination capacity was found between populations ranging from 10% (Ca, red Mediterranean soil) to 75% (Ni, calcareous soil) (Fig. 2). Site and soil of origin as well as their interaction, had a significant influence on germination (Table 2). Populations from calcareous soils and from the westernmost sites germinated significantly better than those from red Mediterranean soils and eastern parts of the study area, respectively. Differences between soil types depended on site of origin, resulting in a significant interaction.

Population differentiation in plant growth was smaller than in germination, but still significant (Table 2, Fig. 2). Differentiation in growth traits did not decrease during the growing season (Appendix S2). The site of origin contributed more to the differentiation than soil of origin. Effect size of site of origin was between 3% of total variation for leaf length and 14% for leaf number whereas effect size of soil of origin was always lower than 1% (Fig. 3). The site × soil interaction explained 1% and 7.7%, respectively, of the model variation. In agreement with the germination results, the westernmost populations from Ni and Mon showed a higher performance in early (leaf length, cumulative leaf length) and late growth traits (tiller length and number, cumulative tiller length, tussock

diameter). The soil type effect was only significant for leaf length being higher in populations from red Mediterranean soils than in those from calcareous soils. In several traits, in particular in leaf length, tiller length and plant diameter, a significant site × soil of origin interaction was detected. For the westernmost sites of origin, growth was higher in populations from red Mediterranean soils whereas no differences or even a higher performance on calcareous soils were found for populations from the eastern and central parts of the study area.

Adaptation to differences in soil conditions, management, and soil moisture

Stone cover around the seedlings significantly increased plant height, tiller number, and thus cumulative tiller length, but not cumulative leaf length (Table 2, Fig. 4). The effect of stone cover was the same for plants from the two soil types, while stone cover had contrasting effects on leaf number and cumulative leaf length as well as on tiller number and cumulative tiller length of plants from the different sites. The populations from SR including the SM populations showed a consistent positive response to stone cover, whereas the response of the Me populations was negative or neutral.

Simulated grazing (clipping) significantly reduced plant height, leaf number, tiller number, cumulative tiller length, cumulative leaf length, and inflorescence number (Table 3, Fig. 5). The negative effect on tiller number was the result of cutting underneath lateral branches that were also counted as tillers. While site × treatment interactions were not significant, several significant soil × treatment interactions were detected. Populations from red Mediterranean soils showed significantly higher compensatory growth resulting in smaller differences in plant height, cumulative tiller length, and inflorescences number between clipped and nonclipped plants.

The test of soil moisture effects on germination in different *B. retusum* populations revealed high germination rates even under dry

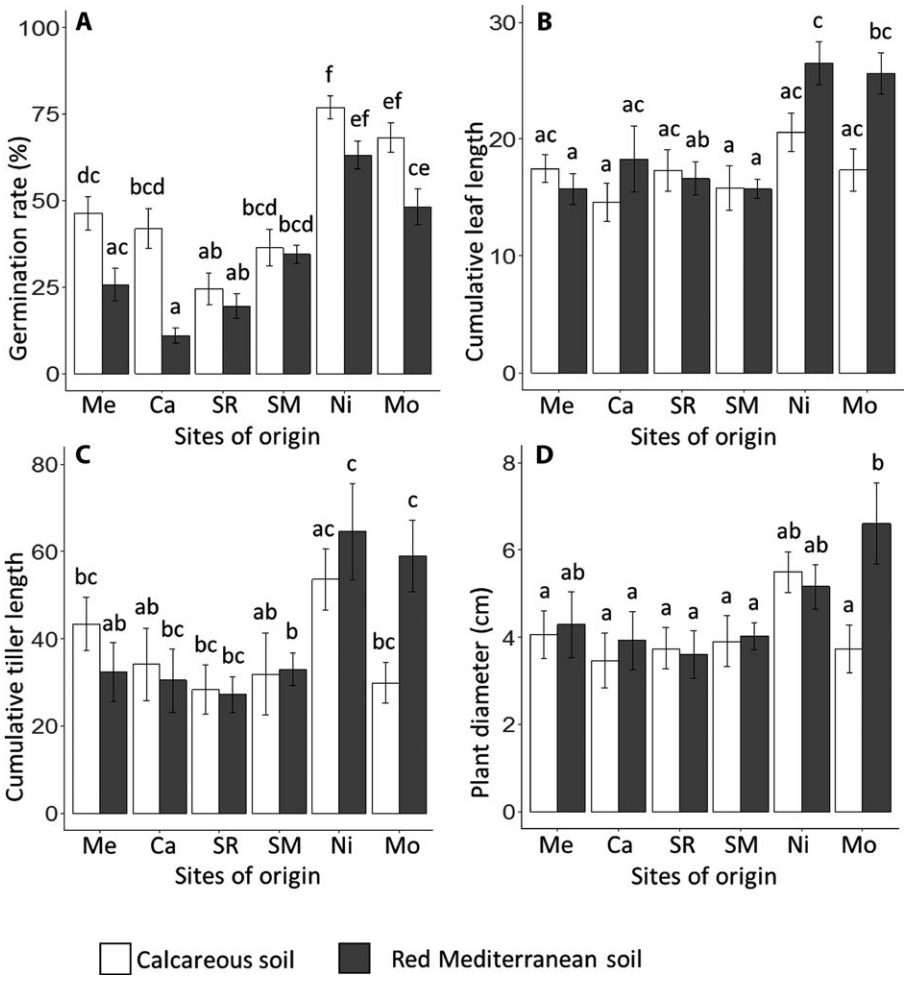


FIGURE 2. Population differentiation decomposed in collection site and soil type within collection site (mean values ±SE, see Table 2 for statistics). A, Final germination. B, Cumulative leaf length. C, Cumulative tiller length. D, Plant diameter. Different lower case letters indicate significant differences ($P < 0.05$).

TABLE 2. Differentiation in *Brachypodium retusum* populations from different sites and soil types, and differential population response to stone cover treatment. *F*-values and significance levels from two-way ANOVA before stone treatment (growth traits: May) and three-way ANOVA afterwards (July) including the stone effect. * $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$; NS, not significant.

		df	Germination	Leaf number	Leaf length	Plant height	Tiller number	Cumulative leaf length	Cumulative tiller length	Plant diameter
Before treatment (May)	Site	5,338	31.41 ****	1.82 NS	11.81 ****	6.62 ****	4.87 ****	8.12 ****	6.98 ***	
	Soil	1,338	36.83 ****	0.00 NS	5.30 **	0.00 NS	0.02 NS	1.05 NS	0.00 NS	
	Soil × Site	5,338	3.08 ***	0.64 NS	6.60 ***	2.06 *	1.76 NS	2.00 *	2.13 *	
After treatment (July)	Site	5,315		1.73 NS	3.99 ***	7.09 ****	6.93 ****	4.89 ****	8.48 ***	4.01 **
	Soil	1,315		0.00 NS	5.07 **	0.37 NS	0.34 NS	1.32 NS	0.38 NS	1.61 NS
	Stones	1,315		1.58 NS	1.09 NS	4.24 **	5.48 **	0.03 NS	5.67 *	4.16 *
	Soil × Site	5,315		0.97 NS	3.02 **	2.18 **	1.33 NS	2.04 *	1.76 NS	2.52 *
	Stones × Site	5,315		2.02 *	0.54 NS	1.56 NS	2.87 **	0.18 NS	2.24 *	0.38 NS
	Stones × Soil	1,315		0.10 NS	1.90 NS	0.59 NS	0.02 NS	1.14 NS	0.05 NS	0.44 NS
	Stones × Soil × Site	5,315		0.49 NS	0.18 NS	2.26 **	0.21 NS	1.18 NS	0.79 NS	0.26 NS

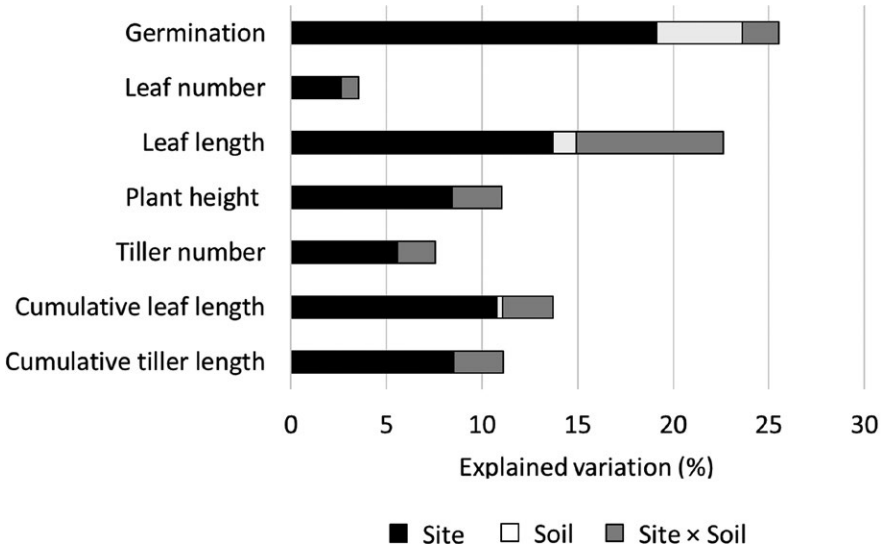


FIGURE 3. Percentage of variation explained by collection site, soil type within collection site and site × soil interaction for growth traits (May).

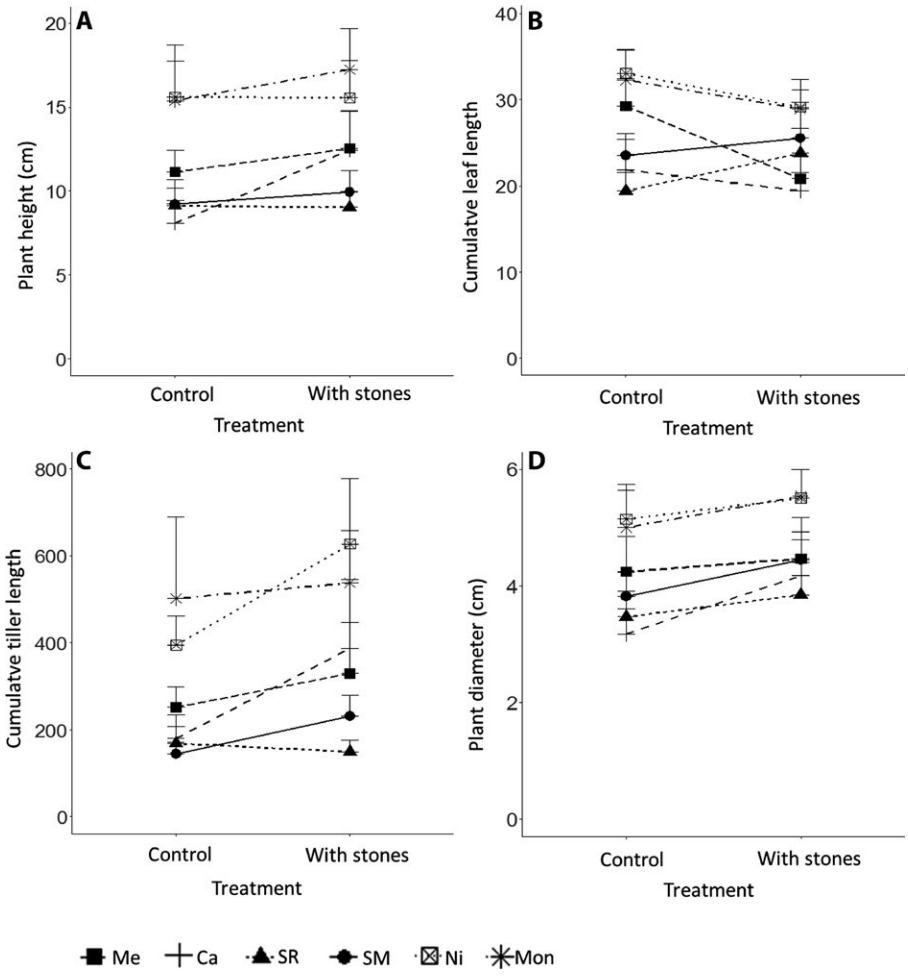


FIGURE 4. Differential response to stone cover (mean values +SE) between *Brachypodium retusum* populations in southern France. A, Plant height. B, Cumulative leaf length. C, Cumulative tiller length. D, Plant diameter.

conditions (0.3% volumetric water content) and a significant decrease of germination under maximum soil moisture (at saturation level: 19.5%, Fig. 6). The highest germination was obtained for intermediate soil moisture levels. With 50% to 80% on average, germination rates were higher than in peat plugs used for the germination test on population differentiation. In agreement with the peat plug germination test, site and soil type effects were significant with a higher germination rate in populations from calcareous soils and from the westernmost sites, respectively (Fig. 6).

The interaction between soil type of origin and soil moisture was not significant and the one between site and soil moisture was only marginally significant suggesting similar adaptation to dry conditions. The marginally significant interaction was explained by a stronger negative response of the Ni populations to drought stress compared with populations from the three other sites.

DISCUSSION

Population differentiation under common conditions

Our common garden study demonstrated genetic differentiation among populations at a regional scale. We found significant differences between populations for germination capacity and several morphological traits such as leaf length, tiller number, and height whereas variation in leaf number per tiller was low. In particular, germination and tiller number are closely linked to seedling recruitment and establishment, thus supporting our hypothesis that different colonization patterns observed at different Mediterranean sites (high: Caturla et al. 2000, Luis et al. 2004; low: Coiffait-Gombault et al., 2012) may result from genetic differentiation.

Site of origin better explained this differentiation than soil type. Independent of soil type, the two eastern populations showed significantly lower germination rates and growth than western populations. The significant effect of site is likely the result of climatic differences between eastern and western parts of the study area although our design does not allow us to distinguish random genetic drift from climatic differences as drivers of differentiation. The sites of the western populations (Ni, Mon) are closer to the Cévennes Mountains and receive more rainfall than those of the Eastern populations (Ceresetti, 2011).

TABLE 3. Differential response in a subset of populations to simulated grazing. *F*-values and significance levels from three-way ANOVA on site, soil (within site) and clipping effects. Vegetative traits measured in spring (May), inflorescence number in June of the second growing season. * $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$; NS, not significant.

	df	Leaf number	Leaf length	Plant height	Tiller number	Cumulative leaf length	Cumulative tiller length	Plant diameter	Inflorescence number
Site	3,132	1.20 NS	1.13 NS	0.27 NS	0.63 NS	1.47 NS	0.64 NS	1.30 NS	2.48 *
Soil	1,132	0.02 NS	9.43 ***	3.16 *	0.05 NS	4.13 **	2.87 *	1.54 NS	3.91 *
Clipping	1,132	12.31 ****	3.19 *	220.98 ****	10.48 ***	11.46 ****	211.72 ****	7.23 **	58.39 ***
Soil × Site	3,132	0.67 NS	2.12 NS	0.81 NS	0.16 NS	2.05 NS	1.18 NS	0.67 NS	1.98 NS
Clipping × Site	3,132	0.66 NS	0.49 NS	0.81 NS	0.02 NS	0.99 NS	1.49 NS	0.94 NS	0.23 NS
Clipping × Soil	1,132	0.08 NS	0.09 NS	3.93 **	2.17 NS	0.12 NS	3.07 *	0.48 NS	3.37 *
Clipping × Soil × Site	3,132	1.12 NS	0.61 NS	1.65 NS	0.76 NS	0.43 NS	1.08 NS	0.40 NS	0.29 NS

Climate is known to be a major driver of population differentiation and adaptation (Linhart and Grant, 1996; Etterson, 2004; Ravenscroft et al., 2014). Water availability and temperature have a particularly strong effect on plant fitness leading to population differentiation in the case of spatial variation (Shah and Paulsen, 2003; Xu and Zhou, 2006). Yuan et al. (2016) found for example that population differentiation in the grass species *Leymus chinensis* was driven by precipitation, temperature, and soil water content. A mainly soil moisture driven differentiation was also shown for *Boechera stricta* (Lee and Mitchell-Olds, 2011). However, the authors also found that isolation by distance due to stochastic processes such as genetic drift considerably contributes to population differentiation. Adaptive differentiation and isolation-by-distance effects often act at the same time on the genetic structure of populations and their contribution to differentiation may be difficult to distinguish (Michalski and Durka, 2012; Durka et al., 2017). Nevertheless, shoot and leaf growth (number and length) were lower in populations from dryer Eastern sites suggesting a selection for growth forms with smaller and less numerous leaves and shoots that are better protected against drought stress (Liancourt and Tielbörger, 2009; Pérez-Ramos et al., 2013). The significant site effects on the cited growth traits were stable throughout the whole growing season and did not decrease with time suggesting a minor influence of environmental maternal effects on our results. Environmental maternal effects may contribute to population differentiation, but particularly affect early traits and vanish during plant development (Roach and Wulff, 1987; Donohue, 2009).

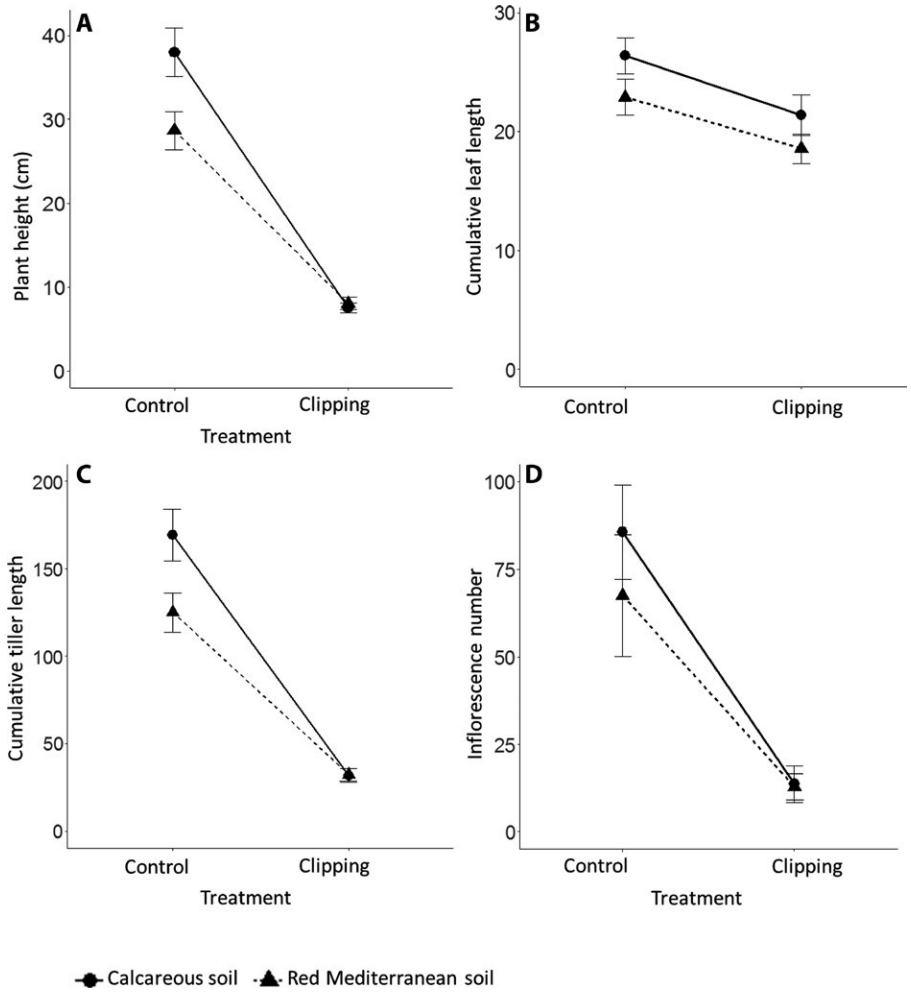


FIGURE 5. Differential response of a subset of populations to simulated grazing (mean values ±SE). Since site × clipping interaction was never significant only soil type × clipping combinations are presented. A, Plant height. B, Cumulative leaf length. C, Cumulative tiller length. D, Inflorescence number.

Soil type is also known to select for population differentiation in plants (Linhart and Grant, 1996; Raabova et al., 2011). In our study, we detected a significant soil type of origin effect on differentiation at a small scale of 10–20 km, but only for germination capacity and leaf length. Germination is under stronger control of environmental maternal effects than growth traits affecting population differentiation in such early traits (Roach and Wulff, 1987; Bischoff and Müller-Schärer, 2010). However, it is also possible that selection for clonal growth under higher grazing pressure of red Mediterranean soils has reduced allocation of resources to seeds compared with less grazed calcareous sites. Although providing some evidence for soil of origin effects, our

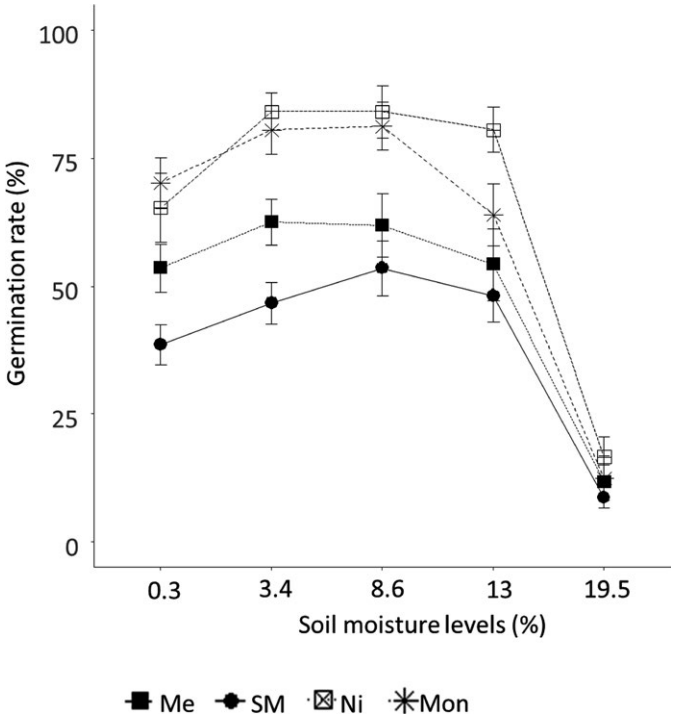


FIGURE 6. Differential germination response of a subset of populations to soil moisture (mean values ±SE). Since the soil of origin x soil moisture interaction was never significant only site of origin effects are presented. Soil moisture ($F_{4,55}$ = 53.71, P < 0.001), site of origin ($F_{3,505}$ = 27.96, P < 0.001), soil of origin ($F_{1,505}$ = 33.86, P < 0.001), site of origin × soil of origin × moisture interaction ($F_{12,505}$ = 1.726).

results do not confirm earlier studies showing a high small-scale differentiation along strong environmental gradients (Lenssen et al., 2004; Bischoff et al., 2006; Raabová et al., 2007; Bischoff and Hurault, 2013). Because no specific barriers prevented pollen dispersal at this scale, gene flow may have counteracted local adaptation to soil type (Kawecki and Ebert, 2004).

Adaptation to differences in soil conditions, management, and soil moisture

Brachypodium retusum populations showed a differential response to stone cover and clipping, and to a lesser degree to soil moisture in the germination phase.

Our study demonstrated a clearly positive effect of stone cover on the growth of *B. retusum*, in particular on plant height, tiller number, and plant diameter. Stone cover increases soil moisture by shading and water condensation, and protects lower stem parts and roots from strong solar radiation and heat (Peters et al., 2008; Buisson et al., 2015). A differential response of Aizoaceae species demonstrated that plants might be locally adapted to type and density of stone cover (Ellis and Weis, 2006). In our study, however, the differential response was not related to the dominant soil types, but to site of origin. Although the texture and depth of calcareous soils and red Mediterranean soils are different, limestone layers and pieces close to the soil surface may have similar effects on microclimate. Thus, the differential response to stone cover cannot be related to differences in local environmental conditions at collection sites.

As expected, clipping largely reduced plant height, tiller number, and leaf number throughout the whole season resulting in a highly reduced reproduction. The effect was less strong in populations from red Mediterranean soils than in populations from calcareous sites suggesting an adaptation to higher grazing intensity. Grazing exerts strong directional selection and may result in genetic differentiation and local adaptation to long-term differences in grazing intensity (Linhart and Grant, 1996; Hufford and Mazer, 2012).

Higher compensatory growth may be one important adaptation of grasses to higher grazing intensity (Sarmiento, 1992). Although we do not exactly know whether our collection sites show such long-term differences, red Mediterranean soils were more grazed in the past than calcareous soils because slopes are less steep and rocky, and soils are more fertile—both of these attributes favor sheep grazing (Verheye and De la Rosa, 2009).

Drought stress reduces germination, and usually plant species show a positive correlation between soil moisture and germination rates (Bochet et al., 2007; Thomas et al., 2010). The ability to germinate fast under moderate-to-low soil moisture may be crucial for species colonizing dry environments (Schütz et al., 2002; Bochet et al., 2007). All tested *B. retusum* populations showed a high germination capacity even under the lowest soil moisture level (–0.18 MPa = 0.3% volumetric water content), but a considerable drop under saturated conditions. The observed germination pattern differs from the results of Bochet et al. (2007) who found a strong decrease in *B. retusum* germination already at water potentials of –0.01 and –0.05 MPa. Although the results may depend on age of seeds and specific experimental conditions this difference suggests genetic variability in germination response to soil moisture. Environmental maternal effects may contribute to population differentiation in germination, but there is little evidence for a contribution to adaptive responses through transgenerational plasticity (Galloway and Etterson, 2007; Bischoff and Müller-Schärer, 2010). In our study, the response to soil water potential was not much different among populations. Only the population of one of the two sites with the most humid climate (Ni) showed a stronger decline under dry conditions suggesting weak adaptation to climate-mediated soil moisture.

CONCLUSIONS

Significant population differentiation was found at a regional scale of 30–200 km. Differentiation between major soil types representing strong environmental differences were found at even smaller scales (10–20 km). However, differentiation between soil types was weaker than differentiation among sites, suggesting a major influence of climate and/or genetic drift on population differentiation.

Stone cover increased *B. retusum* growth and the differential response of populations suggests adaptation to this environmental factor. However, we could not relate this response to the home environment of tested populations. Further experiments are required to identify potential drivers of adaptation to stone cover (density at the surface or in different soil layers). The response to clipping was also clearly different among populations, and the differential response could be linked to the major soil type suggesting that different grazing intensity in the past has selected for different ecotypes. *Brachypodium retusum* showed high germination under dry soil conditions, but there was only weak evidence for a differential response of tested populations. The study demonstrated that

population differentiation may well explain different colonization patterns observed in different Mediterranean regions (Caturla et al., 2000; Luis et al., 2004; Coiffait-Gombault et al., 2012). Because observed differences may largely influence seedling establishment in Mediterranean dry grasslands, plant origin needs to be carefully considered in ecological restoration involving planting or sowing of *B. retusum*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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