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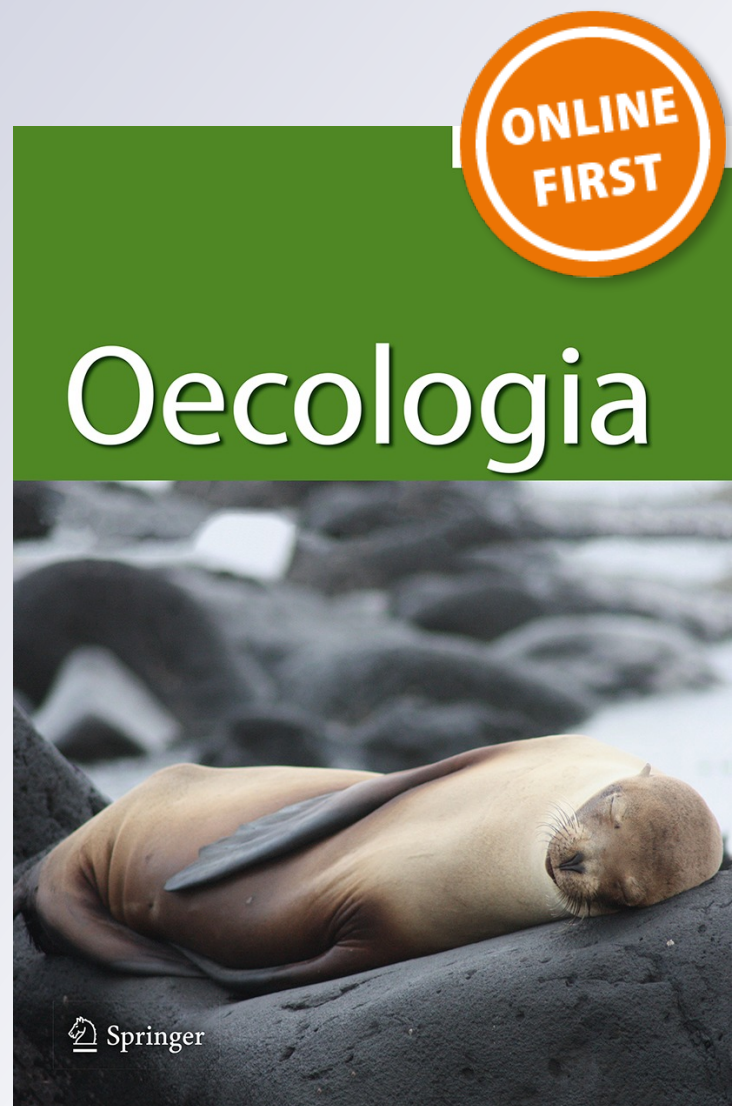
Ana Lázaro-Nogal, Silvia Matesanz, Lea Hallik, Alisa Krasnova, Anna Traveset & Fernando Valladares

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Population differentiation in a Mediterranean relict shrub: the potential role of local adaptation for coping with climate change

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Abstract Plants can respond to climate change by either migrating, adapting to the new conditions or going extinct. Relict plant species of limited distribution can be especially vulnerable as they are usually composed of small and isolated populations, which may reduce their ability to cope with rapidly changing environmental conditions. The aim of this study was to assess the vulnerability of *Cneorum tricoccon* L. (Cneoraceae), a Mediterranean relict shrub of limited distribution, to a future drier climate. We evaluated population differentiation in functional traits related to drought tolerance across seven representative populations of the species' range. We measured morphological and physiological traits in both the field and the greenhouse under three water availability levels. Large phenotypic differences among populations were found under field conditions. All populations responded plastically to simulated drought, but they differed in mean trait values as well as in the slope of the phenotypic response. Particularly, dry-edge populations exhibited multiple functional traits that favored drought tolerance, such as more sclerophyllous leaves, strong stomatal control but high photosynthetic rates, which increases water use efficiency (iWUE), and an enhanced ability to accumulate sugars as osmolytes. Although drought decreased RGR in all populations, this reduction was smaller for populations from the dry edge. Our results suggest that dry-edge populations of this relict species are well adapted to drought, which could potentially mitigate the species' extinction risk under drier scenarios. Dry-edge populations not only have a great conservation value but can also change expectations from current species' distribution models.

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Introduction

It is well known that climate change can have multiple consequences for natural populations, including changes in species' distributions and local extinctions. Plants respond to climate change by migrating to suitable habitats, adapting to the new conditions and by relative quick adjustments via phenotypic plasticity (Bradshaw and Holzapfel 2006; Hoffmann and Sgrò 2011). Relict species are especially vulnerable to climate change (Ohlemuller et al. 2008; Hampe and Jump 2011), as they often have limited opportunities for migration due to geographical isolation in climatic refugia (Dobrowski 2011). Moreover, climate relicts

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are usually composed of small and isolated populations where genetic processes such as genetic drift and founder effects can lead to the loss of genetic variability and to increased inbreeding (Ellstrand and Elam 1993; Arnaud-Haond et al. 2006), which can have a negative impact on individual fitness (Leimu and Fischer 2008). Furthermore, relict species usually experience a specific set of restricted climatic conditions, so small changes in temperature and precipitation patterns could strongly affect their growth and reproduction (Hampe 2005).

Extinction risk of relict species under climate change could be counteracted by high levels of phenotypic plasticity, i.e. the ability of a single genotype to alter its phenotype in response to the environment (Pigliucci 2001), and by differentiation of locally adapted ecotypes (Kawecki and Ebert 2004). Plant populations throughout the entire distribution range of a species are often exposed to different selection pressures, which can lead to develop morphological and physiological adaptations to the local environment, generating ecotypic differentiation in fitness and functional traits (Leimu and Fischer 2008). Water availability, the main selection pressure in Mediterranean ecosystems (Blondel et al. 2010), has led to different morphological (e.g., cushion growth, lignotubers, deep root system) and physiological adaptations [e.g., stomatal closure, increased water use efficiency (WUE), synthesis of sugars and other osmotically active compounds] (Matesanz and Valladares 2014). Rapid microevolution in these functional traits related to drought tolerance, as well as in their plasticity, may alleviate the impacts of ongoing climate change.

Several studies on Mediterranean woody species have documented that populations at the southern edge of their distribution often reach their limit of drought tolerance as seen in reduced growth (Linares and Tiscar 2010), physiological performance (Peguero-Pina et al. 2007) or reproduction and establishment (Hampe 2005). The expected increased aridity in the Mediterranean (IPCC 2012), together with a predicted increase in precipitation heterogeneity (D'Odorico and Bhattachan 2012), has been argued to more negatively affect southern limit populations compared to populations growing in the center of the distribution under more mesic conditions (Peguero-Pina et al. 2007). However, some studies have reported that populations growing under more xeric habitats could be adapted to water-limited conditions and might respond even better to climate change than populations in mesic habitats (Heschel et al. 2002; Kawecki 2008). Relict plants are natural laboratories and fit models to test how natural plant populations will respond to climate change (Hampe and Petit 2005). Moreover, the great antiquity of most relict species renders them important for the conservation of genetic and phylogenetic diversity (Hampe and Petit 2005; Hampe and Jump 2011).

We have studied *Cneorum tricoccon* L. (Cneoraceae), a western Mediterranean endemic shrub of limited and declining distribution (Traveset 1995a; Lázaro-Nogal et al. 2013), which is considered a relict species of the Tertiary that evolved under tropical conditions (Raven 1973). It has been categorized as vulnerable (IUCN), with human activities and climate change as major threats for the species' survival. Its distribution has diminished in the last few decades, partly due to habitat loss in coastal areas, and many current populations show a low proportion of seedlings and saplings, suggesting low establishment success (Traveset et al. 2012). Previous studies have shown vulnerability to aridity of some populations of this species (Lázaro-Nogal et al. 2013), as well as signs of low genetic variability and high inbreeding ($H_O = 0.004$–0.36; $H_E = 0.004$–0.48; $F_{IS} = -0.02$–0.88) (Lázaro-Nogal et al., unpublished data) as measured with polymorphic microsatellites markers (García-Fernández et al. 2012).

The aims of this study were to assess population differentiation in functional traits related to drought response, as well as estimate population vulnerability under predicted climate change. We selected seven populations across the species' range from locations that were representative of the pronounced aridity gradient, and measured functional traits in both natural and controlled conditions. In situ measurements allowed us to assess phenotypic differentiation and performance under natural conditions. To further assess adaptive population differentiation in response to drought and vulnerability to drier climate, we then applied three distinct, ecologically meaningful water treatments simulating current and future water availability conditions. We measured a wide suite of morphological and physiological traits to assess population differentiation in both mean trait values and their plasticity. Our specific hypotheses were: (1) in natural conditions, populations at the dry edge of *C. tricoccon* distribution range will show lower performance (in terms of growth, physiology and reproduction) than those established on more mesic sites, as has been previously shown for rear-edge populations of Mediterranean plants (Hampe and Petit 2005); (2) however, because populations at the dry edge have been exposed to long-term drought, they may have evolved efficient adaptations to low water availability, such as lower SLA, strong stomatal control, higher iWUE increase and enhanced ability to accumulate sugars as osmolytes; and (3) under the expected aridity increase, dry-edge populations could be less vulnerable than more mesic populations if they have not already reached their physiological limits and they are able to display plastic responses to novel conditions.

Materials and methods

Study species

Cneorum tricoccon L. (Cneoraceae) is an evergreen shrub, usually less than 1 m tall, with small, yellow flowers that have three stamens and carpels, and indehiscent, red fruits with three locules. It is endemic to the western Mediterranean area, being found in the Balearic Islands, some localities in the eastern and southern Iberian Peninsula and south-eastern France, plus Sardinia, Giannutri and Tuscany in Italy. *Cneorum tricoccon* is an andromonoecious, insect-pollinated species (Traveset 1995a). Seed dispersal is mediated by endemic lizards (*Podarcis lilfordi*, *P. pityusensis* and *P. siculus*), and by introduced carnivorous mammals (*Martes martes* and *Genetta genetta*) (Traveset 1995b). *Cneorum tricoccon* is an ideal study system for our purpose because it is a relict species of the Tertiary, which evolved under a warm, tropical climate with rainy summers. Currently, it is geographically limited to the coastal maquis within climatic refugia with mild temperatures and high air humidity coming from the sea breeze.

Study sites

The study was performed in seven populations spanning most of the species' distribution range and covering a pronounced climatic gradient of precipitation and temperature (Fig. 1). Selected populations were large and well established (>50 adult individuals), and sites were neither managed nor surrounded by highly disturbed areas. The study sites ranged from a maximum mean annual precipitation of 755 mm to a minimum of 382 mm (climatic data were obtained from WordClim; Table 1). To characterize the abiotic and biotic environment of the study sites, we measured the slope and estimated the percentage of bare soil, cover of rocks, woody plants and herbs on ten randomly selected transects (2 × 10 m) per study site. In addition, we identified surrounding vegetation in each location. We also measured photosynthetically active radiation (PAR) at

Fig. 1 *Cneorum tricoccon*'s distribution range and location of sampled populations. Study site codes in Table 1
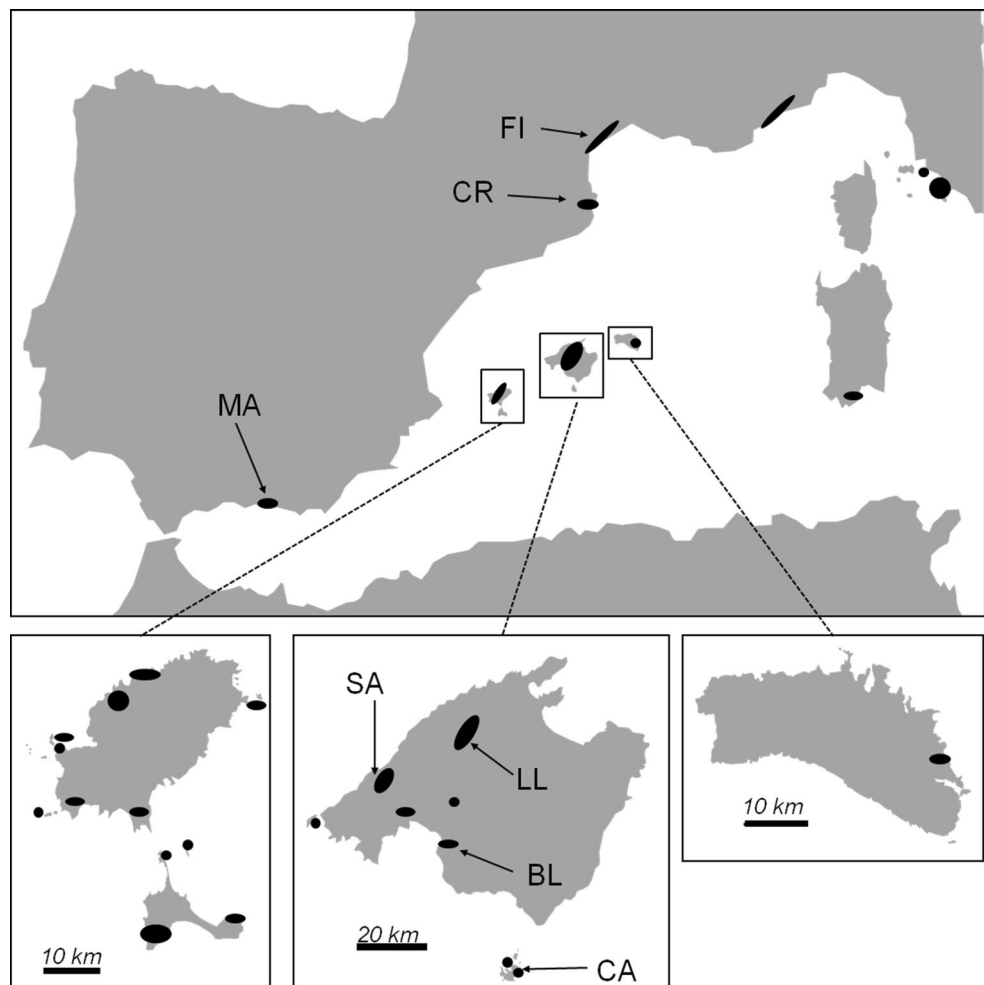
Table 1 Location, biotic and abiotic characterization of the seven study populations of *Cneorum tricoccon*

Population	Lluc	Fitou	S'Arboçar	Cap Creus	Cap Blanc	Cabrera	Maro
Code	LL	FI	SA	CR	BL	CA	MA
Latitude (°)	39.79	43.54	39.68	42.26	39.38	39.15	36.76
Longitude (°)	2.86	3.78	2.54	3.27	2.78	2.95	−3.85
Altitude (m a.s.l.)	693	63	443	147	92	58	175
Mean annual precipitation (mm)	755	730	613	594	509	463	382
Precipitation driest month (mm)	13	24	10	21	6	5	2
Precipitation seasonality range (cv)	41	31	42	32	49	52	63
Mean anual temperature (°C)	13.3	13.9	14.7	15.1	16.4	16.7	17.6
Mean temperature warmest month (°C)	25.8	28.4	27.0	27.3	28.6	28.6	30.8
Temperaure anual range (°C)	22.2	26.9	22.3	22.8	22.3	21.9	24.3
Population size	±5000	±1000	±2000	±200	±300	±200	±500
Slope (%)	65.9 (6.01)	10.6 (0.84)	9.8 (2.48)	–	0.2 (0.42)	19.00 (10.93)	33.1 (1.28)
PAR (μmol m^{-2} s^{-1})	1694.9 (394.50)	1369.5 (168.50)	1286.7 (439.10)	–	1438.5 (257.50)	1588.5 (283.50)	1624.5 (271.50)
Bare soil mean (%)	9.00 (5.16)	15.5 (2.83)	10 (3.33)	–	27.5 (3.53)	19.5 (8.56)	21.0 (3.16)
Cover rocks mean (%)	12.00 (7.74)	24.0 (3.16)	20.00 (3.33)	–	22.5 (3.53)	25.00 (12.35)	20.0 (3.34)
Cover herbs mean (%)	24.00 (5.16)	22. (5.37)	15.00 (5.77)	–	9.0 (4.59)	11.75 (11.03)	29.0 (5.67)
Cover woody plants mean (%)	55.00 (4.08)	38.5 (5.29)	55.00 (5.77)	–	41.0 (4.59)	43.75 (8.09)	30.0 (3.34)
Surrounding vegetation	*Quercus ilex, Pinus halepensis, Rhamnus ludovici-salvatoris, Smilax aspera var balearica, Rubia balearica*	*P. halepensis, Juniperus oxicedrus, Olea europaea var. sylvestris, Cystus albidus, Rosmarinus officinalis, R. alaternus*	*P. halepensis, Q. ilex, Pistacia lentiscus, Arbutus unedo, C. albidus, Erica arborea, Daphne gnidium*		*O. europaea var. sylvestris, P. lentiscus, Cystus monspeliensis, C. albidus, R. officinalis*	*P. lentiscus, O. europaea var. sylvestris, J. phoenicea, Phyllerea angustifolia, Ephedra fragilis*	*Chamaerops humilis, O. europaea var. sylvestris, R. lycioides, Asparragus horridus, S. aspera, Tymus communis*

Climatic data were obtained from WordClim. Standard deviations are shown in parentheses

cv coefficient of variation, *PAR* photosynthetic active radiation

population level (in ten selected transects) and at microhabitat level (0.2 m to every sampled individual) with a portable PAR leaf-clip (FMS2; Hansatech, UK). All populations occurred on calcareous soils (Table 1).

Field measurements

Field measurements were performed in six of the seven populations. Population CR was used in the greenhouse experiment but was excluded from the field due to logistic limitations. In April–August 2010, we measured maximum plant height, projected crown diameter in the longest dimension and trunk diameter in 80 randomly selected adult plants per population (80 plants × 6 populations = 480). Due to the logistic requirements of the traits collected, we selected a subsample of individuals in which we measured different ecophysiological traits. In all cases, sample sizes exceeded those recommended so as to obtain an appropriate indication about the values for the trait of interest (Cornelissen et al. 2003). We measured morphological leaf traits, i.e., specific leaf area (SLA), leaf size and leaf thickness, in 36 randomly selected plants per population (36 plants × 6 populations = 216). Furthermore, we measured physiological traits, i.e. stomatal conductance and photochemical efficiency (Fv/Fm) in 15 plants per population (15 plants × 6 populations = 90) (sample size for each trait is presented in the Online Resource 1). The latter traits were measured in spring and again in summer 2010 in order to assess differences in physiological traits due to summer drought. Selected individuals for ecophysiological measurements met the following criteria: (1) neighboring plants grew at least 3 m apart to minimize intense resource competition, (2) individuals had similar height and crown diameters (close to the mean for each population) to reduce the potential confounding factor of plant size, and (3) sampled individuals grew in habitats with similar irradiances, specifically with photosynthetically active radiation (PAR) during the central hours of the day between 1200 and 1600 μmol photons $m^{-2}s^{-1}$. All leaf traits were measured in five healthy and fully expanded leaves collected from the exposed upper parts of the canopy. SLA was measured using the ratio of the area of one side of a fresh leaf divided by its oven-dried mass (Cornelissen et al. 2003). Leaf areas were scanned and analyzed with the image analysis software ImageJ (Rasband 2009). Leaf thickness was measured with a dial thickness gauge (Mitutoyo, Aurora, IL, USA). Stomatal conductance was measured with a leaf porometer (SC-1; Decagon Devices, Pullman, WA, USA) from 1000 to 1200 hours in four leaves per plant. Photochemical efficiency (Fv/Fm) was measured with a portable pulse-modulated fluorometer (FMS2; Hansatech) from 0900 to 1200 hours in three leaves adapted to the

dark for 30 min with leaf clips. Minimal (Fo) and maximal (Fm) fluorescence were measured, and these values were used to calculate photochemical efficiency (Fv/Fm) as Fv/Fm = (Fm − Fo)/Fm, where Fv is the difference between Fm and Fo (Maxwell and Johnson 2000).

In June–August 2010, matching the fruiting peak of the species in each location, we collected fruits from 30 randomly selected mother plants (genotypes) per population. In order to sample a broad genetic diversity and potential micro-environmental heterogeneity within populations, we selected plants growing at least 5 m from their neighbors. Fruits were manually depulped and seeds were stored in a cool and dry environment. Previously, we had measured the length of the three seed axes to determine seed size according to $V = 3/4 \ \Pi \ a \ b \ c$ (a, b, c being the values for each axis) in five seeds per mother plant.

Common garden and experimental treatments

In 16–22 September 2010, 100–200 seeds from 30 genotypes from the seven populations were stratified for 24 h in water with sodium hypochlorite 0.5 %, then rinsed with distilled water, and finally sown in one individual tray (60 × 40 × 12 cm) per family with a 4:2:1:1 mixture of coco peat, sterilized peat moss, vermiculite (Projar, Spain) and expanded clay balls (Hnos. Aguado, Spain). On 10 February 2011, healthy seedlings of similar heights were individually transplanted into 1.2-L pots filled with the same soil mixture. We randomly selected 20 genotypes from the initial 30 and 2–3 seedlings per genotype, in order to sample a broad genetic diversity within each population. The final sample was 378 plants (7 populations × 20 genotypes/population × 2–3 seedlings/genotype) (see Online Resource 1 for details on the experimental design). We followed a complete randomized design and changed each pot's location once a week in order to minimize the effect of micro-environmental variation (Zar 1999). All seedlings were grown for 9 months in a greenhouse under favorable conditions of high water and nutrient availability, in order to study drought response in well-established individuals, and trying to minimize potential maternal effects (Roach and Wulff 1987; Galloway 2001). On 10 June 2011, we started a drought experiment by growing plants under progressively reduced water availability during 2.5 months (June–August 2011). Field capacity for our soil mixture was determined for a random sub-sample of 21 pots (3 per population) at the beginning of the experiment. All pots were weighed twice a week, and tap water was added to compensate evapotranspiration losses until the desired water availability level (see below), was reached in order to maintain the same soil water content (SWC) in all pots within the same treatment. Our rationale for

the implementation of the water treatments is as follows. The influence of the plant on the actual water balance of the pot-soil–plant system is slightly different compared to natural conditions. Water saving or water spending plants differentially affect water availability in the pot, which is more influenced by the plant than in the field. Therefore, to implement the desired water level in each pot, we applied slightly different amounts of water to each pot based on individual demands estimated by periodic weightings. With this approach, we maintained common water availability conditions (the same SWC) for all plants independently of their water strategy.

We considered three levels of water availability: (1) moist conditions, where plants were kept at field capacity (68.67 ± 0.67 % of soil water content, SWC), (2) early drought conditions, where plants were kept at 40 % of field capacity (27.2 ± 0.53 % SWC) and (3) late drought conditions, 10 % of field capacity (6.8 ± 0.43 % SWC) (temporal dynamics of SWC during the water stress experiment are provided in the Online Resource 2). The moist and early drought treatments were designed to mimic contrasting water availability levels comparables with current climatic conditions. While the moist treatment simulated optimum water conditions, the early drought is equivalent to a typical Mediterranean locality in the summer period (Ogaya and Peñuelas 2007). In turn, the late drought treatment was designed to mimic a drier climate scenario, reducing the soil water content by 20.4 % in comparison with the early drought treatment, as has been done previously in similar Mediterranean habitats (Sardans et al. 2013) which experimentally induced the increase in aridity predicted by future climate change scenarios (IPCC 2012). These levels of water availability, designed here as treatments, correspond to three timepoints along the drought experiment: at the onset (Moist), after 1 month (early drought) and after 2.5 months (late drought). Considering different timepoints along the extent of the drought period allowed evaluating the dynamics of the measured functional traits, which can reveal firstly acclimation and improved stress tolerance and finally plant senescence. It is unlikely that the different starting points of the water treatments involve a confounding factor in the determination of phenotypic differences among treatments, given the relatively short time period in which the treatments took place (2.5 months) in the context of the life cycle of the species (life span >50 years). Temperature and photosynthetically active radiation (PAR) were recorded every 10 min during the whole drought experiment with a HOBO H08-006-04 data logger (Onset, Pocasset, MA, USA). Mean daily temperature for this period was 22.5 °C; mean maximum and minimum temperature were 39.6 and 15.1 °C, respectively; and mean daily PAR was 31.6 mol m^{-2} d^{-1}. These conditions were largely similar to field conditions (Lázaro-Nogal et al. 2013).

Data collection in the common garden

Sample size

Growth-related traits were measured in 54 individuals per population (2–3 seedlings per genotype) and physiological traits were measured in 30 individuals per population (1–2 seedlings per genotype). Midday stem water potential (Ψ_{MD}), morphological leaf traits and soluble carbohydrate content were measured in 12 individuals per population (1 seedling per genotype) (sample size for each trait is presented in the Online Resource 1). Again, these sample sizes aimed to obtain a robust phenotypic characterization of each population, accounting for the logistic requirements for data collection in each case (see Cornelissen et al. 2003).

Growth-related traits

We measured plant height before the onset of the drought experiment at two time periods, 10 and 70 days after the first germinated seed was censused, and during the drought experiment in the moist, early drought and late drought conditions (days 100, 130 and 160, respectively). Plant height was measured as the elongation above the cotyledons to the node of the most recent fully expanded leaf. RGR was calculated as RGR = (Ln H$_{t2}$ – Ln H$_{t1}$) T$_{1/2}^{-1}$, where H$_{t1}$ and H$_{t2}$ are plant height (mm) in the early and late censuses, respectively, and T (month) is the time elapsed between censuses. RGR is considered a sound surrogate for fitness in many studies of woody plants (e.g., Ramírez-Valiente et al. 2010a; Salgado-Luarte and Gianoli 2010) and in our species, plant size is also a good surrogate for fitness since it is highly correlated with fruit crop ($R^2 = 0.193$; $P < 0.001$; data from 342 individuals sampled in the same study populations).

Physiological traits

We measured photosynthetic rate, stomatal conductance and instantaneous water use efficiency (iWUE). Measurements were performed from 1000 to 1500 hours on 5 comparable sunny days. Water use efficiency (iWUE) was calculated as the ratio of photosynthetic rate and stomatal conductance. Physiological traits were measured on one fully expanded leaf of a primary branch per plant using a Licor 6400 (LI-COR, Lincoln, NE, USA). Leaves were exposed to an atmospheric CO_2 concentration of 400 µmol CO_2 mol^{-1} (using the built-in controller) and saturating light of 1400 µmol m^{-2} s^{-1}. Stomatal ratio was set to 1 and gas flow to 400 µmol s^{-1}. Measurements were performed at 25–26 °C and a relative humidity of 45–55 %. When the leaf did not completely cover the chamber, leaf tracings

were scanned and photosynthetic surface area was calculated using ImageJ (see above). Midday stem water potential (Ψ_{MD}) was measured with a Scholander pressure chamber (Scholander et al. 1965) from 1300 to 15 1500 hours.

Morphological leaf traits

We measured specific leaf area (SLA) and leaf size, the former calculated as described above, one fully expanded leaf from a primary branch per plant being harvested and immediately scanned. Leaves were then oven-dried (at 65 °C for 48 h) and weighed to obtain dry mass. Leaf areas were calculated using ImageJ.

Soluble carbohydrates

To assess soluble carbohydrate content (glucose, fructose, sucrose), leaf samples were ground, freeze-dried to prevent natural enzymes from sugar degradation, and weighed. Soluble sugars were extracted with 80 % ethanol for 24 h at 80 °C. Glucose, fructose and sucrose contents were determined enzymatically with consecutive assay using hexokinase and glucose-6-phosphate dehydrogenase (H8629 Sigma), phosphoglucose isomerase (P5381 Sigma) and invertase (I4504 Sigma) from baker's yeast (*S. cerevisiae*). NADPH absorption at 340 nm was measured with UV-2550 Spectrophotometer (Shimadzu, Tokyo, Japan).

Statistical analyses

Prior to all analyses, we performed correlation matrices among all measured traits in both the field and the common garden in order to eliminate redundant traits and minimize consequences associated to multiple testing (see Online Resource 3 and 4 for correlation matrices in the field and common garden, respectively). We reduced the range of variables by using a mixed approach combining correlation values and ecophysiological information related to drought adaptation provided by the measured traits. For the field dataset, we retained plant height, trunk diameter, SLA, fruit crop, stomatal conductance and Fv/Fm (i.e. 5 out of the 13 measured traits; see Online Resource 3); for data from the greenhouse common garden, we maintained plant height, RGR, photosynthetic rate, iWUE, Ψ_{MD}, SLA, SSC, and glucose/fructose and glucose/sucrose ratios (i.e. 3 out of the 9 traits; see Online Resource 4). In other words, we retained those traits that had moderate correlation values but have been repeatedly shown to provide key information on adaptation to drought. For the remaining traits, we performed one-way ANOVAs to assess differences among populations in the field measurements with population as

the main fixed effect (see Quinn and Keough 2002). Two-way ANOVAs were used to test for differences among populations and season (measurements were taken in spring and summer) on stomatal conductance and photochemical efficiency in field conditions; the model included population and season as main effects and its interaction.

For the traits measured in the common garden, we performed preliminary multivariate analyses of variance (MANOVA) on three sets of ecologically meaningfultraits: growth-related traits (which included plant height and RGR), physiological traits (photosynthetic rate and iWUE) and carbohydrates (SSC, glucose/fructose and glucose/sucrose) examining the fixed effect of population, treatment and their interaction. MANOVAs were evaluated using the Wilk's lambda test criterion (Scheiner 2001). Following a significant population, treatment and/or interaction effect in the MANOVAs, protected ANOVAs (those only performed when the multivariate analyses yields a significant result; Scheiner 2001) were carried out for each trait individually. The univariate models tested for the fixed effects of population, treatment and their interaction, and the random effect of genotype (nested within population). A significant effect of population indicates genetically based phenotypic differences; a significant effect of treatment indicates plasticity for the trait; and the interaction population × treatment indicates that differences among populations are not consistent from one environment to another (i.e. population differentiation for plasticity). These models were repeated with mixed models using restricted maximum likelihood (REML), testing for the fixed effects of population, treatment and interaction, and the random effect of genotype. Significance of the random term (variance components) was tested via likelihood ratio tests, by comparing the full model (including fixed and random factors) with the reduced model (dropping the random factor). The results of the REML mixed models and the mixed ANOVAs were virtually identical. Furthermore, individual analyses were performed within each treatment to test for the fixed effect of population, followed by post hoc Tukey's HSD. Differences in plasticity among populations were evaluated by comparing the slopes of the reaction norms via *t* tests (Zar 1999) when there was a significant population × treatment interaction. Normality and homogeneity of variance of the dependent variables were tested prior to analyses by means of the Kolmogorov–Smirnov and the Levene's test, respectively.

To test for local adaptation in the sampled populations, RGR (as a fitness proxy) in moist and early drought conditions was regressed against mean annual precipitation at each population site. Pearson's correlation coefficients between precipitation and RGR were calculated for both the early drought and moist treatment. Formal tests of local adaptation involve reciprocal transplants among sites,

which can sometimes be logistically unfeasible (Kawecki and Ebert 2004). A valid and repeatedly used approach involves investigation of local adaptation in controlled common garden experiments that simulate the diverse conditions found in natural habitats (Parker et al. 2003; Matesanz et al. 2012). These studies provide evidence for local adaptation when certain populations have higher overall fitness in conditions similar to the populations' sites of origin than populations from contrasting conditions.

REML analyses were performed using the package *lme4* (Bates et al. 2015) in R 3.2.1. All other statistical analyses were performed using Statistica 6.0 (StatSoft, Tulsa, OK, USA).

Results

Population differentiation in the field

Populations differed in all phenotypic traits measured in the field (Table 2; Fig. 2). Plants in the xeric populations BL and CA were on average 29 % smaller and tended to show lower trunk diameters than those in mesic ones. However, the most xeric population, located in the southern limit of the species distribution range (MA) was an exception, as plants were of similar size as those in mesic populations (Fig. 2a, b). More xeric populations (BL, CA, MA) had 59 % lower SLA than more mesic ones (Fig. 2c). By contrast, more mesic populations had 83 % higher fruit crop, than the xeric ones BL and CA (Fig. 2g), with the exception again of the most xeric population (MA), which showed a fruit crop similar to mesic ones. Stomatal conductance also differed among populations and seasons, with interactions among these factors (Table 2). Stomatal conductance was proportional to population aridity in spring, whereas it showed a similar, strong decrease in summer in all populations (Fig. 3a). The effect of season was 86 % larger than that of population. Photochemical efficiency (Fv/Fm) also differed among populations and seasons with a significant

population × season interaction (Table 2). Photochemical efficiency (Fv/Fm) decreased slightly with aridity in spring, although the summer decrease was consistently greater in more xeric populations (Fig. 3b). The effect of season was 74 % larger than that of population. We did not find differences in seed size among populations ($F = 1.948$, $P = 0.076$).

Population differentiation in the greenhouse common garden

Broad phenotypic differences were found among populations and drought treatments for growth-related, physiological and morphological leaf traits and carbohydrates (Table 3; MANOVA results are provided in the Online Resource 5). These patterns of plasticity to water availability differed among populations for all traits (Table 3; significant population × treatment terms). Overall, no significant differences were found among genotypes within populations.

Growth-related traits

The populations bearing the tallest plants in field conditions (LL, SA and MA) showed the largest seedlings at the end of greenhouse experiment (Fig. 4). Height and RGR were consistently affected by drought treatment (Table 3; MANOVA results on the Online Resource 5); however, the slope of the reaction norm (i.e., the graphical representation of phenotypic plasticity across environments) differed among populations (Table 3). In moist conditions, individuals from more mesic populations (LL, FI, SA, CR) were on average 25 % larger than those from more xeric populations (BL, CA, MA). However, under early and late drought, they showed reduced growth whereas individuals from more xeric populations continued to increase in height (Fig. 4). RGR decreased in all populations both under early and late drought. However, more xeric populations showed flatter reaction norms than more mesic ones,

Table 2 ANOVA of functional traits measured in field conditions in *Cneorum tricoccon* plants across six representative populations of the species' range, in two seasons (spring and summer)

	n	Population (Num $df = 5$) Den df	F	P	Season (Num $df = 1$) Den df	F	P	Population × season (Num $df = 5$) Den df	F	P
Height	80	446	24.46	<0.001	–	–	–	–	–	–
Trunk diameter	80	446	7.41	<0.001	–	–	–	–	–	–
SLA	36	209	134.95	<0.001	–	–	–	–	–	–
Fruit crop	80	446	19.381	<0.001	–	–	–	–	–	–
Stomatal conductance	15	200	26.76	<0.001	200	194.99	<0.001	200	4.2779	<0.001
Fv/Fm	15	200	14.817	<0.001	200	56.187	<0.001	200	4.054	<0.001

F ratios, *P* values, numerator (Num *df*) and denominator (Den *df*) degrees of freedom are shown.

SLA specific leaf area, *Fv/Fm* photochemical efficiency
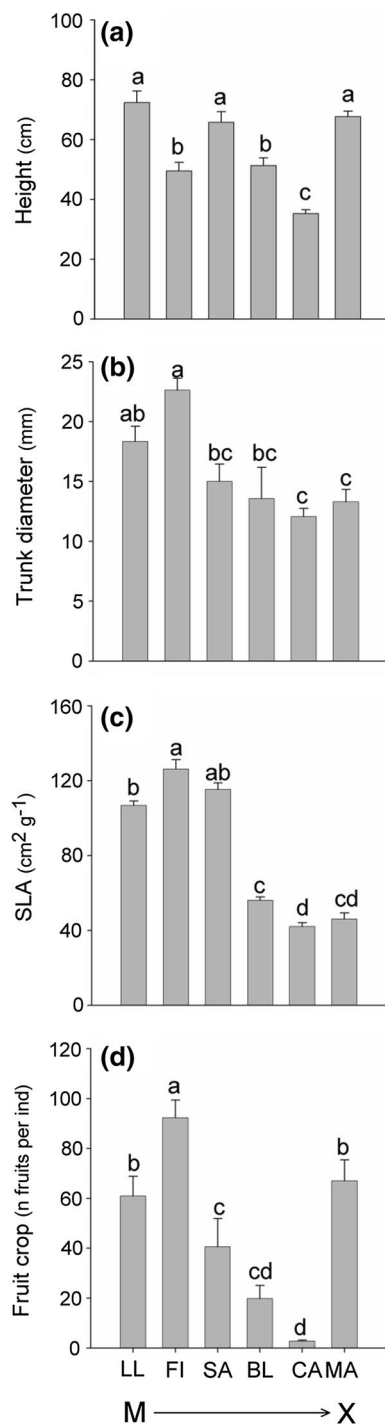
Fig. 2 Population differences in functional traits measured in the field along an aridity gradient: **a** plant height, **b** trunk diameter, **c** specific leaf area (SLA), **d** fruit crop. Population mean ± SE are shown for 80 individuals per population in (**a**, **b**, and **d**), and 36 genotypes per population in (**c**). *Different letters above the columns* indicate significant differences after Tukey's HDS test. *M* mesic, *D* dry. Study site codes in Table 1
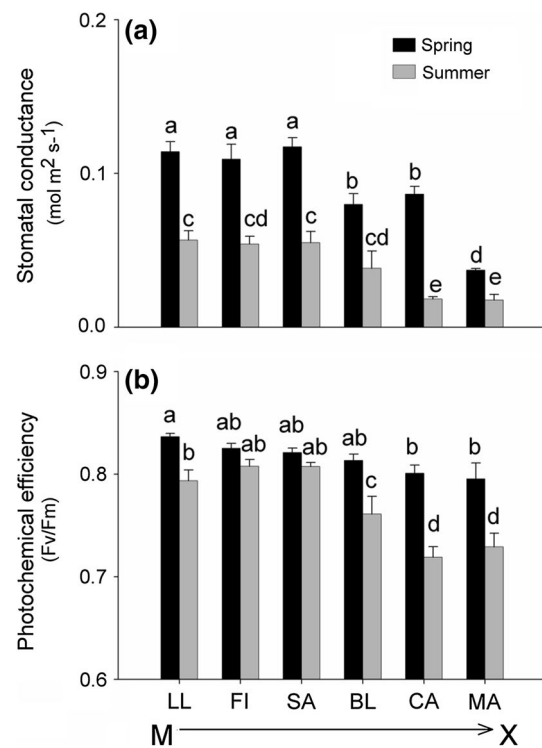
Fig. 3 Population differences in **a** stomatal conductance and **b** photochemical efficiency (Fv/Fm) in spring (*black columns*) and summer (*gray columns*) measured in the field. Population mean ± SE are shown for 15 individuals per population. *Different letters above the columns* indicate significant differences after Tukey's HDS test. *M* mesic, *D* dry. Study site codes in Table 1

which decreased deeply the RGR under early drought. This is revealed by more mesic populations showing an 83–97 % RGR decrease, in comparison with a 17–47 % in more xeric populations). No significant differences were found among populations under late drought (Fig. 5a). We found intra-population differences for plant height but not for RGR [Table 3; genotype(population) terms].

Physiological traits

Photosynthetic rate and iWUE were affected by drought in all populations (Table 3; MANOVA results in Online Resource 5). However, differences were found in plasticity patterns of these functional traits among populations (Table 3). Individuals from populations on more mesic sites (LL, FI, SA, CR) showed greater decrease in photosynthetic rate under early drought than individuals from more xeric populations (BL, CA, MA), the change in xeric and mesic populations being −37 to 9 and −133 to −94 %, respectively. BL and CA showed flat norms of reaction

Table 3 ANOVA of functional traits in *Cneorum tricoccon* plants from seven populations exposed to different water availability treatments in a greenhouse common garden

	N	Population (Num $df = 6$)			Treatment (Num $df = 2$)			Pop × treat (Num $df = 12$)			Genot (pop) (Num $df = 133$)		
		Den df	F	*P*	*Den df*	F	*P*	*Den df*	F	*P*	*Den df*	F	*P*
Growth-related traits													
Plant height	54	133	10.34	**<0.001**	980	17.49	**<0.001**	980	2.29	0.007	54.89	2.90	**<0.001**
RGR	54	133	90.17	**<0.001**	980	632.12	**<0.001**	980	16.70	**<0.001**	54.89	0.93	0.694
Physiological traits													
Photosynthetic rate	30	133	12.12	**<0.001**	476	212.17	**<0.001**	476	6.24	**<0.001**	2.78	0.73	0.984
iWUE	30	133	22.88	**<0.001**	476	17.22	**<0.001**	476	4.59	**<0.001**	2.78	1.21	0.076
Ψ_{MD}	12	133	8.18	**<0.001**	231	78.25	**<0.001**	231	2.47	**<0.001**	–	–	–
Morphological leaf traits													
SLA	12	133	144.43	**<0.001**	231	10.74	**<0.001**	231	3.02	**<0.001**	–	–	–
Carbohydrates													
SCC	12	133	3.37	**<0.001**	231	26.13	**<0.001**	231	1.88	0.04	–	–	–
Glucose/fructose	12	133	1.21	0.31	231	10.96	**<0.001**	231	0.54	0.88	–	–	–
Glucose/sucrose	12	133	2.12	0.06	231	25.81	**<0.001**	231	1.95	0.04	–	–	–

F ratios, *P* values, numerator (Num *df*) and denominator (Den *df*) degrees of freedom are shown. Bold type indicates significance ($P < 0.001$)

RGR relative growth rate, *iWUE* instantaneous water use efficiency, Ψ_{MD} midday stem water potential, *SLA* specific leaf area, *SCC* soluble carbohydrates content
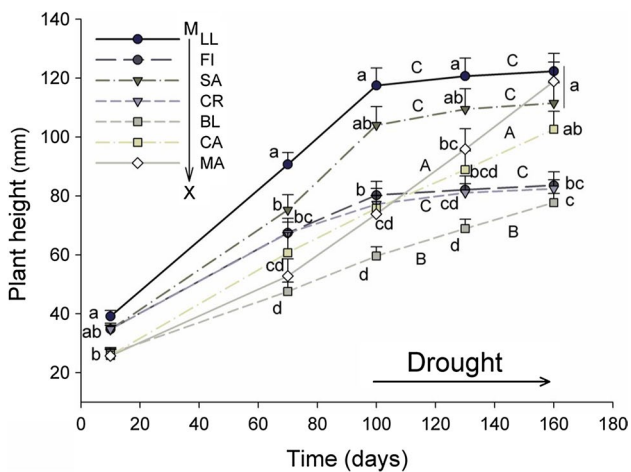
Fig. 4 Differences in growth before and during simulated drought in populations of *Cneorum tricoccon*. Populations in the legend are ordered following the aridity gradient from *M* (mesic) to *X* (xeric). Population mean ± SE are shown for 56 individuals coming from different mothers. *Different letters* indicate significant differences within each measurement date after Tukey's HDS test. Study site codes in Table 1

(Fig. 5b). Likewise, individuals from more mesic populations decreased iWUE in early and late drought whereas those from more xeric populations increased iWUE in early drought and decreased it in late drought, the most xeric population increasing it ca. 40 % in contrast with an −51 to 4 % change in the more mesic populations. Instantaneous WUE values in xeric populations were consistently higher than those in mesic ones (Fig. 5c). No intra-population differences for photosynthetic rate and iWUE were found [Table 3; genotype(population) term].

Individuals from all populations decreased Ψ_{MD} under early and late drought, but they did it differently (Table 3). All populations expressed similar phenotypes in moist and early drought conditions except MA, which showed a greater Ψ_{MD} decrease in early drought than the other populations (e.g., it decreased 76 % Ψ_{MD} in comparison with the 28 % decrease of the most mesic population). BL and CA also showed a strong decrease in Ψ_{MD} in late drought (Fig. 5d). All populations from more xeric sites reached relatively low Ψ_{MD} without cavitation symptoms.

Morphological leaf traits

SLA values differed significantly among populations (Table 3). Populations from more xeric sites (BL, CA, MA) showed lower SLA than populations from more mesic sites (LL, FI, SA, CR). We have found slightly differences among populations in their response to water availability treatments (Table 3; Fig. 5e). SLA slightly decreased in
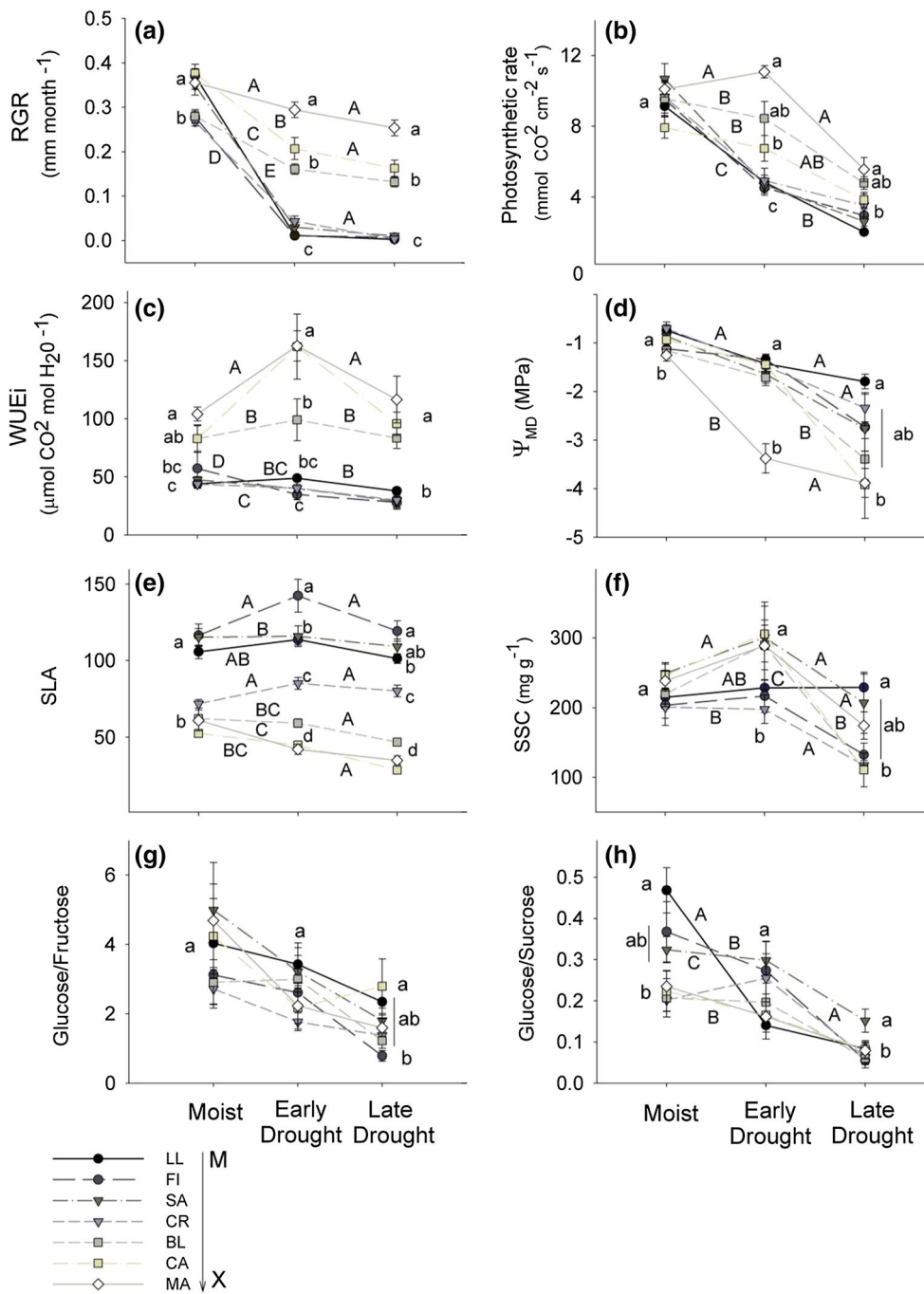
and MA increased photosynthetic rate in early drought. Photosynthetic rate decreased in all populations under late drought, although individuals from the most xeric population (MA) showed higher rates than those from mesic ones (LL, FI, SA, CR) with those from the other xeric populations (CA and BL) showing intermediate decreases

Fig. 5 Differences in drought responses for several functional traits in populations of *Cneorum tricoccon:* **a** *RGR* relative growth rate, **b** photosynthetic rate, **c** *iWUE* instantaneous water use efficiency, **d** Ψ_{MD}, midday stem water potential, **e** *SLA* specific leaf area, **f** *SCC* soluble carbohydrates concentration, **g** glucose/fructose ratio, **h** glucose/sucrose ratio. Populations in the legend are ordered fol- lowing the aridity gradient from *M* (mesic) to *X* (xeric). Population mean ± SE are shown. *Different capital letters* indicate significant differences in the slope of the reaction norm among populations. *Lowercase letters* indicate significant differences among populations within treatment after Tukey's HDS test. Study site codes in Table 1

more xeric populations (−44 to −4 % of change) while it increased in more mesic ones under early drought (5–18 % of change) and finally decreased 1–38 % under late drought. SLA in the greenhouse experiment were similar to the values observed in field (Fig. 3d) suggesting not just an SLA environmental response but also a genetic component.

Soluble carbohydrates

The sum of measured soluble carbohydrates (SSC) was affected by treatment, but the effect size depended on the population (Table 3; Online Resource 5). In general, early drought resulted in the increase of SSC while late drought produced the opposite effect. This pattern was more visible for populations on the three xeric sites (BL, CA, MA) and in one mesic site (FI). In contrast to all other populations the reduction of SSC under late drought was not observed for the most mesic population (LL) (Fig. 5f). Drought also affected ratios of different carbohydrates. Glucose to fructose and glucose to sucrose ratios decreased in response to drought. The most mesic population (LL) demonstrated the rapid drop down of glucose to sucrose ratio already under early drought (69 % of decrease whereas the others decreased 7–31 %). This population had the highest monosaccharides content compared to all other populations in moist conditions and the lowest under early drought being the most affected by early drought compared to all other populations (Fig. 5h). We found a significant interaction between population and treatment for glucose/sucrose ratio, while for glucose/fructose this interaction was not significant (Fig. 5g, h; Table 3).
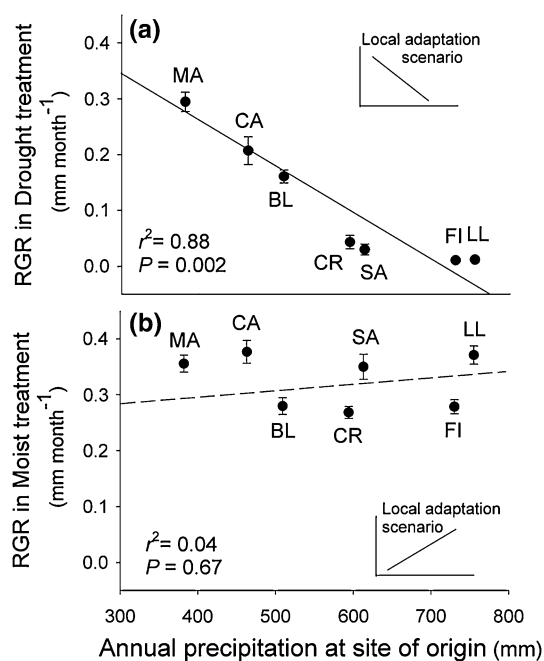
Fig. 6 Local adaptation analyses in seven populations of *Cneorum tricoccon*. Correlations between RGR (our fitness proxy) in **a** early drought and **b** moist treatment with mean annual precipitation at each population site. Population mean ± SE (n = 56 per population), R^2 and P values are shown. Study sites codes in Table 1

Test of local adaptation

There was a strong negative correlation between RGR (our fitness proxy) in the drought treatment and annual precipitation ($R^2 = 0.88$, $P = 0.002$), i.e. populations in sites with low annual precipitations had higher RGR under drought than population with higher annual precipitations (Fig. 6a). Conversely, the correlation between RGR in the moist treatment and annual precipitation of the origin site was not significant ($R^2 = 0.03$, $P = 0.671$), i.e. all populations showed high RGR in moist conditions (Fig. 6b).

Discussion

Our results suggest that populations at the southern limit of *C. tricoccon*'s range are likely adapted to dry conditions. This may provide such populations with a head start to cope with the predicted increase in aridity compared to populations growing under mesic conditions Significant population differentiation was found in the field along the distribution range of *C. tricoccon*. Individuals from populations on more xeric sites showed ecophysiological traits aimed at reducing evaporative water losses and enhancing water use efficiency, such as more sclerophyllous leaves and strong stomatal closure. Similar results were obtained in the common garden, suggesting that the differences observed in natural conditions are not just a plastic response to environmental conditions but have a genetic basis. These phenotypic differences suggested that populations may be locally adapted, and agree well with the described trait syndrome repeatedly shown to be adaptive under low-water conditions (Ackerly 2004; Dudley 1996; Ramírez-Valiente et al. 2010b). Studies on a number of Mediterranean woody species have reported that populations at the drier portion of their distribution are strongly limited by water availability (Hampe and Petit 2005; Linares and Tiscar 2010). In contrast, our results showed that growth and reproduction of the dry-edge population (MA) seemed not to be highly limited by aridity. Individuals in this population showed similar size and fruit crop as those under mesic conditions, thus suggesting adaption to dry conditions, although we cannot exclude that these differences were due to other factors such as different population establishment time.

Drought adaptation was also confirmed in the greenhouse experiment. Populations in more xeric sites showed higher RGR (our fitness proxy) in environments with similar water availability to their origin sites compared to populations from mesic habitats ('local vs. foreign criteria'; sensu Kawecki and Ebert 2004). This finding is in agreement with other studies of semiarid plants (see, e.g., Dudley 1996; Heschel et al. 2002; Ramírez-Valiente et al. 2010b). Conversely, the lack of a positive association between RGR

under moist conditions and precipitation at the study sites suggests that populations are not locally adapted to moist conditions. Under moist conditions, all populations showed high fitness, which is consistent with *C. tricoccon* requirements of humidity and mild temperatures of early Tertiary species. We suggest that divergent selection and thus local adaptation can be stronger when resources are limited, such as in the more xeric populations (Dudley 1996; Ramírez-Valiente et al. 2010a, b). In this context, mesic populations may be exposed to less intense selection pressures than xeric ones, which could explain the lack of local adaptation to moist conditions. Alternatively, local adaptation to dry conditions can be the result of the population's responses to the stronger historical environmental change occurring in xeric sites compared to mesic ones.

The morphological and physiological adjustments found could enhance growth and performance under water limiting conditions, which also supports our argument that xeric populations are drought-adapted. All *C. tricoccon* populations showed a consistent, thigh stomatal closure under drought to minimize water loss and avoid hydraulic failure, matching observations for the species under field conditions (Lázaro-Nogal et al. 2013) and studies of other Mediterranean shrubs (Gulías et al. 2009; Quero et al. 2011). Stomatal closure usually impairs carbon assimilation (Ehleringer 1993), but *C. tricoccon* populations from xeric sites were able to maintain and even increase photosynthetic rates under early drought. This might explain why xeric populations strongly increased iWUE under moderate drought, which is common in semiarid annuals (Dudley 1996; Heschel et al. 2002) and perennial species (Donovan and Ehleringer 1994; Casper et al. 2005). Most expectations on iWUE increase under water stress conditions are focused on stomatal closure instead of on carbon assimilation increase (Ehleringer 1993). However, in more xeric populations of *C. tricoccon*, the increase in iWUE was due not only to stomatal closure but also to the ability to keep stable or even increase carbon assimilation, particularly in the dry-edge population (MA). The ability to increase iWUE under water-limited conditions is an adaptive mechanism observed in several Mediterranean plants (Pías et al. 2010; Gimeno et al. 2012) that could result from local adaptation (Matesanz and Valladares 2014; Ramírez-Valiente et al. 2010a). Populations from mesic sites could be vulnerable to prolonged droughts since carbon starvation becomes a serious threat when stomatal conductance is reduced with no photosynthetic compensation (McDowell et al. 2008).

All *C. tricoccon* populations decreased their water potential (Ψ_{MD}) under water-limited conditions. Contrary to what we expected, the dry-edge population showed the greatest decrease in Ψ_{MD}. Low Ψ_{MD} under drought is common in species that maintain high stomatal conductances

(McDowell et al. 2008), which was not the case of the populations from more xeric sites, CA and MA. This unexpected result could be explained by the different scales involved in each trait: while stomatal conductance is a leaf level process (Ehleringer 1993), stem water potential (Ψ) corresponds to the water status of the whole plant (Zimmermann 1983). Though low Ψ enhanced the probability to embolize and to die through hydraulic failure (Sperry and Tyree 1988), individuals from *C. tricoccon*'s dry-edge population were able to diminish Ψ_{MD} without suffering cavitation, since they maintained high photosynthetic rates even under drought. Studies in semi-arid woody plants have reported that cavitation vulnerability varies widely among species (Maherali et al. 2004) and among populations (Kavanagh et al. 1999). A study done with *Eucalyptus* showed that susceptibility to cavitation may be a function of both morphology and physical modifications subsequent to previous drought (Price et al. 2004). High photosynthetic rates characteristic of the *C. tricoccon*'s dry-edge population even under low Ψ_{MD} could be explained by osmotic adjustments that allow maintaining positive cell turgor through accumulation of solutes, as in other semi-arid plants (Warren et al. 2011).

Leaf soluble carbohydrates analyses illustrated the complexity of plant responses to drought duration and intensity (Rosas et al. 2013). In general, it is hypothesized that mild drought stress could lead to the acclimation and improved stress tolerance while severe stress depletes reserves (Niinemets 2010). Our results confirm this hypothesis. Leaf soluble carbohydrate pools increased in response to early drought while prolonged drought induced the decline in major soluble sugar pools. This pattern was particularly clear for populations from xeric sites while the most mesic population (LL) also maintained elevated levels of carbohydrate pools under late drought. Increasing content of sugars—which can act as osmolytes helping to preserve membrane integrity and enhancing cell homeostasis under drought—leads to improved stress tolerance (Hüve et al. 2006). Soluble carbohydrates are not merely carbon and energy sources but they also act as important signaling molecules controlling gene expression and having important post-translational effects on metabolic enzymes activity (Pego et al. 2000; Price et al. 2004). Plant ability to cope with changing environments is largely governed by the dual operation of soluble sugars as energy source and signaling molecules regulating plant growth, development and metabolism as well as stress resistance (Rolland et al. 2006). Several works have suggested that the ratio of glucose to sucrose might be more important for signaling than the absolute concentrations of glucose and sucrose (Balibrea Lara et al. 2004). In accordance with this suggestion, our results also show a clearer response of carbohydrate ratios than absolute contents to drought. While it is

extremely difficult to distinguish the role of single adjustment within this highly integrated network, it appears that populations differ in their response to drought mainly by sucrose content as the parameters involving sucrose (SSC and glucose/sucrose ratio) had significant interaction between population and treatment.

Alongside the contrasting patterns of drought response among populations, the population differentiation found in the mean value of some functional traits can also contribute to local adaptation in *C. tricoccon* populations. Individuals from more xeric populations had overall lower SLA than individuals from mesic populations, a pattern observed both in the field and in common garden conditions. Several studies have reported that more sclerophyllous leaves provide fitness benefits under water-limited environments since sclerophylly avoids water loss, maintaining photosynthetic rates and consequently allows a more conservative water use (Ackerly et al. 2002; Dudley 1996; Ramírez-Valiente et al. 2010a). The low plasticity expressed in important functional traits such as SLA, may suggest a strong selection on the mean trait values, i.e. phenotypic canalization, a conservative syndrome already reported in certain Mediterranean species (Valladares et al. 2002). We observed high levels of phenotypic plasticity in some traits (e.g., photosynthetic rate and soluble carbohydrates) while others were canalized (e.g., SLA). Plasticity may be favored over canalization depending firstly on the scale and frequency of environmental variation and secondly on the natural costs and limits of plasticity for each trait (Weinig 2000); in some cases, the metabolic cost of plasticity may increase and exceed the benefit of maintaining it (van Kleunen and Fischer 2005). Our results suggest that a combination of both canalization in some traits (mainly structural) and plasticity in others (mainly physiological) would represent an advantage in response to climate change.

A reduction in genetic diversity and increased inbreeding are expected in small and isolated populations, which may outweigh selection and reduce the possibility to adapt to the climatic stress (Arnaud-Haond et al. 2006; Ellstrand and Elam 1993). However, our results suggest that dry-edge populations are well adapted to drought. If current adaptation to dry conditions provide populations in more xeric sites with a head start for further adaptation to increasing aridity, we could expect mesic populations of the species to be potentially more vulnerable to climate change than xeric ones. Nevertheless, we cannot discard the possibility that mesic populations will be able to adapt to the predicted drought if they are subjected to dry conditions for several generations.

Our study highlights the importance of dry-edge populations, especially in relict species of limited and regressive distribution, since they may preserve rare alleles and gene combinations that are important for adaptation to extreme environmental conditions (Hampe and Petit 2005). These dry-edge populations could counteract the habitat loss expected under climate change. Hence, intraspecific differentiation in niche breath and phenotypic plasticity should be incorporated in species distributions models to render more realistic scenarios.

Author contribution statement ALN, FV and AT conceived and designed the experiment; ALN conducted field work and the greenhouse experiment; LH and AK performed soluble carbohydrates analyses; ALN and SM analyzed the data; ALN, SM, FV and AT wrote the manuscript; other authors provided editorial advice.

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