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ORIGINAL PAPER

# Phenotypic plasticity blurs ecotypic divergence in the response of *Quercus coccifera* and *Pinus halepensis* to water stress

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**Abstract** The Mediterranean evergreen woody plants *Quercus coccifera* and *Pinus halepensis* grow in a range of environments where selection by drought, heat and high irradiance can drive genetic and phenotypic differentiation of populations. However, the role of these stresses in filtering out maladaptive genotypes remains unknown. We hypothesize that this filtering is an important process for woody Mediterranean species due to their low phenotypic plasticity reported in previous studies. We have studied the response of saplings of *Q. coccifera* and *P. halepensis*, originating from two contrasting populations (a rock outcrop and a garrigue formation), to water stress. Isozyme characterization of genetic diversity was done to determine whether populations were genetically distinct. Water response analysis was based on water relations, gas exchange, chlorophyll *a* fluorescence, pigment content, antioxidant status and morphological and structural parameters. Ecotypic differentiation was found for both *Q. coccifera* and *P. halepensis* populations, with a higher population isozyme similarity and a higher frequency of dominance of a few genotypes at the rock outcrop in both the species. *P. halepensis* exhibited small but significant differences between populations for plastic responses to water, with lower phenotypic plasticity in saplings from the rock outcrop. Although it was not found in *Q. coccifera,* this pattern suggests that ecotypic differentiation rendering stress-tolerant ecotypes involves a decreased plasticity. Phenotypic plasticity was not high but it explained over 75% of the total variability among individual plants. Thus, and although evidence for ecotypic divergence was found in both the species, saplings were plastic enough to blur ecotypic differentiation.

**Keywords** Drought · Ecotype · Genetic variability · Mediterranean species · Phenotypic plasticity

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## Introduction

Evolution in response to environmental heterogeneity can lead to two alternatives: specialized adaptations to a subset of environment conditions or generalized adaptations to a broad range of environments (Bradshaw 1965; Valladares et al. 2000b). These two outcomes are not mutually exclusive and their relative importance strongly depends on both the traits analyzed and the particular environmental conditions. Empirical data show that some traits present plastic variation, whereas other traits are less plastic and exhibit heritable

variation and population divergence. So the conventional a priori expectation would be that populations within a species will have some plastic traits that are similar and some traits where the degree of plasticity varies among populations.

High levels of adaptive phenotypic plasticity are associated with a generalist strategy, so adaptation across a range of environments is conferred by an ability to plastically adjust the phenotype to each set of conditions (Balaguer et al. 2001; Lortie and Aarssen 1996). Ecotypic differentiation involving a reduced-phenotypic plasticity has been associated to specialists, those limited to a restricted distribution range or to a particular habitat-, which exhibit narrow tolerance to environmental changes (van Tienderen 1997). Selection by abiotic factors can drive genetic differentiation in the functional traits of populations occurring in contrasting environments (Linhart and Grant 1996). In support to this, ecotypic differentiation has been observed in many tree species for a wide range of physiological and structural parameters (Abrams 1994; Bailey and Harrington 2006; Balaguer et al. 2001; Bogdan et al. 2004; Dodd et al. 1998; Maherali et al. 2002; Treseder and Vitousek 2001, among others). But plants are well known for their capacity to acclimate to a variety of environments by means of phenotypic plasticity, which involves morphological and physiological changes of a given genotype to varying environmental conditions (Bradshaw 1965; Schlichting and Pigliucci 1998). Phenotypic plasticity has been well described for the response of plants to light (Balaguer et al. 2001; Gianoli 2004; González and Gianoli 2004; Navas and Garnier 2002; Sleeman and Dudley 2001; Valladares et al. 2000a, b), to nutrients (Navas and Garnier 2002; Robinson and Rorison 1988; Valladares et al. 2000a) and, to a lesser extent, also to water (Castro-Díez et al. 2006; Gianoli 2004; Navas and Garnier 2002; Sleeman and Dudley 2001) .

Water is considered to be the most important factor limiting production, growth, development and distribution of plants in Mediterranean ecosystems (Acherar and Rambal 1992). Water availability varies not only daily and seasonally, but also at various spatial scales, so species occupying a large geographical range or a variety of contrasting habitats must cope with a wide range of water availabilities. The environmental heterogeneity of the Mediterranean region leads to plants dwelling in this region to frequently cope with contrasting conditions. For example, nutrients and water availabilities are low in rocky areas, and consequently competition for these resources is high, whereas competition for light is weak in these locations since the scattered leaves are exposed to intense radiation most of the time (Matthes-Sears and Larson 1999). In contrast, on better soils, nutrient and water availabilities are higher and leaves are partially to fully shaded, as competition for light is high. Species that occupy large geographical ranges or a variety of habitats within a given region deal with all these contrasting environmental conditions either by ecotypic differentiation, genetic polymorphism or phenotypic plasticity (Abrams 1994; Schlichting and Pigliucci 1998). Plants growing in the Mediterranean region undergo relatively long drought periods and it has been shown that they are capable of developing a range of mechanisms to survive under situations of severe water deficit, heat and high irradiance (Gratani et al. 2003; Martínez-Ferri et al. 2000). Although plasticity has been suggested to be limited in Mediterranean woody plants (Valladares et al. 2007), it is not well-known whether the adaptations observed are either the result of a plastic response to the environment or the result of genetic differentiation.

In this work, the performance of saplings of the pioneer Aleppo pine (*Pinus halepensis* Mill.) under water stress was compared to that of the late-successional angiosperm kermes oak (*Quercus coccifera* L.). Both the Mediterranean species have a wide geographical distribution with *Q. coccifera* dominating many garrigue areas, characterized by dense shrub formations and *P. halepensis* colonizing many areas from sea-level and small mountains to exposed and arid rock outcrops (Martínez-Ferri et al. 2000; Terradas 1999). On exposed rock outcrops, both the species form a stunted and widely spaced scrub reaching a height of less than 2 m. The aims of this study were (a) to identify the existence of ecotypic differentiation between *Q. coccifera* and *P. halepensis* populations from two contrasting environments, and (b) to determine whether different ecotypes show divergence in their phenotypic plasticity in response to water availability. Saplings of both the species from a garrigue and a rock outcrop populations were grown in greenhouse conditions under well-watered and water-stressed conditions. A number of physiological and structural parameters were determined together with phenotypic plasticity in response to water and isozyme variability of the original populations. We hypothesize that genetic polymorphism and selection of the most adaptive genotypes are crucial mechanisms in the ecotypic differentiation of populations of these species, since phenotypic plasticity of Mediterranean woody plants has been shown to be low (Balaguer et al. 2001; Valladares et al. 2002a, 2007). Additionally, we expected lower plasticity in plants originating from the population under the most adverse conditions (rock outcrops) as a corollary of the specialization to cope with extreme abiotic stress (Van Tienderen 1997, Valladares et al. 2007).

## Materials and methods

### Seed collection and experimental design

Seeds of *Q. coccifera* and *P. halepensis* were collected from ten mother trees per species in each site, selected at

random in an area of *ca*. 1,000 $m^2$, in two natural populations growing in contrasting environments on the Iberian Peninsula: a continental rock outcrop ('rock') at Cañada de Verich (40°52′N 00°07′W, Teruel, Spain); and a continental garrigue-type formation ('garrigue') at Mata de Olmos (40°51′N 00°32′W, Teruel, Spain). The populations of these species in these areas are quite small due to a combination of factors: land fragmentation, habitat degradation, climatic adversity and poor soil conditions. The populations sampled were representative of these conditions, and even though the number of individual per population was low due to logistic constraints, they were collected in the same way in both sites, so levels of genetic similarity among individuals of the same populations are expected to be alike. Both sites were separated by 50 km and exhibited a Mediterranean climate with a summer drought (period of low-soil moisture) longer than 3 months per year. Mean annual rainfall was higher at the garrigue site than at the rock site, but was not statistically significant (Table 1). Mean annual temperature was also slightly higher at the garrigue site. Both sites also differ in the slope (60% at the rock site and 0% at the garrigue site). As a consequence of the low-soil availability at the rock site, trees were smaller than those of the garrigue site. Soil nitrogen was analyzed using the Kjeldhal method (Harwite 1980) and soil P was determined following the phosphomolibdo-vanadate method (MAFF 1986). Soil organic matter was determined following the method of Schnitzer (1982). All determinations were made with ten replicates in each site.

Seedlings, issued from collected seeds, were grown in plastic pots (5 $dm^3$) containing 4:4:1:1 (v/v) compost, vermiculite, perlite and sand, with slow release N:P:K (14:13:13) fertilizer containing micronutrients (Osmocote, Scotts Miracle-Gro Company, OH, USA). When they were 1-year-old, 20 seedlings per species and origin were assigned randomly to each of 2 water treatments, fully irrigated and drought conditions for two additional years. Then, six to ten plants per species and origin were selected for the experiment. Treatments for the water-stress trial were imposed from January 1999 to July 2001 (30 months). The timing of irrigations (frequency and duration) was based on the soil-water potential monitored in each treatment with the use of granular matrix sensors (Watermark Soil Moisture Sensors, Model 200X, Irrometer Inc., Riverside, CA) and tensiometers placed 15 cm deep. The two treatments consisted of blocks irrigated so as to maintain no-water-stress (control plants) and water-stress conditions. No-water-stressed plants were maintained at −50 kPa, whereas water-stressed plants were maintained at −400 kPa, mimicking the soil water conditions found in summer in the field. Sensors were read hourly, thus in the case soil water potential would drop below these values, irrigation automatically started, applying 5 and 10 ml water in water-stressed and no-water-stressed plants, respectively. All the traits were measured, and samples were taken, at the end of the experiment.

**Table 1** Characteristics of the continental garrigue type formation (garrigue) and continental rock outcrop (rock) where seeds for production of plants were collected

| | Garrigue | Rock |
|---|---|---|
| Area ($m^2$) | ≈1,000 | ≈1,000 |
| Mean annual rainfall (mm) | 465 ± 58 a | 383 ± 35 a |
| Mean annual temperature | 14.3 ± 1.57 a | 12.3 ± 1.37 a |
| Slope (%) | 0 | 60 |
| Soil depth (m) | 0.65 ± 0.15 a | 0.03 ± 0.01 b |
| Tree height (m) ($n$ = 10) | 3.25 ± 1.05 a | 0.97 ± 0.38 b |
| Tree crown diameter (m) ($n$ = 10) | 4.3 ± 1.07 a | 1.14 ± 0.36 b |
| Soil pH ($n$ = 10) | 7.99 ± 0.03 a | 8.04 ± 0.03 a |
| Organic matter (%) ($n$ = 10) | 9.19 ± 0.59 a | 12.57 ± 1.47 b |
| N (%) ($n$ = 10) | 0.53 ± 0.03 a | 0.63 ± 0.06 b |
| P (ppm) ($n$ = 10) | 5.57 ± 0.79 a | 8.25 ± 1.53 b |

Annual rainfall and temperature correspond to the 1990–2002 period. Data are the mean ± SE. Different letters in the same row indicates significant differences ($P < 0.05$)

## Stem-water potential and water content

Stem-water potential was measured at predawn and midday in two twigs per plant with a pressure chamber (Skye Instruments Ltd., UK) as described by Scholander et al. (1965). Total water content was calculated as (FW − DW)/FW in leaves, stems and roots after determining fresh (FW) and dry weight (DW). Dry weight (DW) was quantified after drying the samples in an oven at 70°C until constant weight. Measurements were conducted at predawn (03.00–04.00 h solar time) and at midday (12.00–14.00 h solar time).

## Gas exchange measurements

Leaf exchange measurements were done in two fully expanded current-year leaves per sapling with a portable, open-flow gas exchange system (ADC Bioscientific Ltd., UK) equipped with an IRGA-porometer connected to a conifer cylindrical chamber PLC4C (ADC Bioscientific Ltd., UK). Measured variables were net photosynthetic rate ($A$, μmol $CO_2$ $m^{-2}$ per second), stomatal conductance (gs, mmol $H_2O$ $m^{-2}$ per second) and transpiration rates ($E$, mmol $H_2O$ $m^{-2}$ per second). Air and leaf temperatures in the chambers were controlled by a Peltier unit and were maintained within 1–2°C of ambient air. All results were expressed on a projected leaf area basis, which was determined with a portable leaf area meter AM100 (ADC Bioscientific Ltd., UK). Projected leaf areas of *P. halepensis*

were corrected by multiplying with $\pi/2$ (Cregg 1992). Measurements were conducted in the morning (0800–1000 hours solar time) and at midday (1200–1400 hours solar time).

### Chlorophyll fluorescence

Chlorophyll fluorescence emission was measured with a PAM-2000 portable fluorometer (Heinz Walz, Effeltrich, Germany) in three, current-year, fully expanded leaves per sapling. In the early morning, the minimum-fluorescence yield $F_0$ of leaves adapted to darkness throughout the night was determined under weak red modulated light. The leaf was held in the leaf clip holder of the fluorometer at a standard distance from the optic fiber probe and a weak far-red (735 nm) pulse was sent to fully oxidize the electron transport chain. The maximum-fluorescence yield $F_m$ of the dark-adapted leaves was reached by exposing photosystems to saturating pulse (0.8 s) of white light. Predawn values of maximal ($F_m$) and minimal fluorescence ($F_0$) were used to calculate maximal photochemical efficiency of PSII ($F_v/F_m$). The $F_m$ values were also used to calculate diurnal non-photochemical quenching [$qN = (F_m - F_m')/(F_m - F_0')$; Buschmann 1995]. Diurnal variation in quantum yield of noncyclic electron transport ($\Phi_{PSII}$), photochemical quenching (qP) and photochemical efficiency of the open reaction centers of PSII ($F_v'/F_m'$) were calculated according to Genty et al. (1989). For quenching analysis, minimal-fluorescence yield of a pre-illuminated sample ($F_0'$) was assessed in leaves darkened immediately after every saturation pulse and subsequently exposed to far-red light for 5.5 s. Measurements were conducted in the morning (0800–1000 hours solar time) and at midday (1200–1400 hours solar time).

### Pigment analyses

Two leaf samples per sapling were collected, wrapped in aluminum foil, quick-frozen in liquid nitrogen and stored at −80°C until pigment determination in the laboratory. Chlorophylls and carotenoids were extracted from 10 mg leaf aliquots using 2.5 ml DMSO (dimethyl-sulphoxide) saturated with $CaCO_3$ (Barnes et al. 1992). Tubes containing the reaction mixture were incubated for 40 min at 65°C in the dark (shaking at regular intervals) and then the extracts were allowed to cool to ambient temperature before diluting to 1:1 with fresh DMSO. Absorbance of extracts was then read spectrophotometrically (Uvikon 922, Kontron Ltd., Switzerland) at 480, 665.1 and 649.1 nm using 1 ml quartz-glass cuvettes, calibrated against a blank containing DMSO saturated with $CaCO_3$. The concentrations of chlorophyll *a* ($C_a$) and *b* ($C_b$) as well as total carotenoids ($C_{x+c}$) were calculated using equations of Wellburn (1994).

### Determination of antioxidant enzyme activities and isozyme analyses of leaf peroxidases

Two leaf samples per sapling were collected, wrapped in aluminum foil, quick-frozen in liquid nitrogen and stored at −80°C until determination in the laboratory. Plant frozen material (0.2 g) was homogenized in 4 ml of extraction buffer containing 66 mM $KH_2PO_4/Na_2HPO_4$ pH 7, 0.1% (v/v) Triton X-100 and 1 mM EDTA (ethylenediaminetetraacetic acid), with 0.6 g of insoluble polyvinylpolypyrrolidone using a Mikrodismembrator (Braun Ltd., Germany). The homogenate was filtered through nylon gauze and centrifuged at 20,000*g* for 10 min at 4°C. The supernatant was collected and used for assays of enzyme activities and peroxidase isozymes.

Two different extracts per leaf sample were used for enzymatic analysis. Standard enzymatic assays were performed spectrophotometrically in a total volume of 1 ml at 25°C (Uvikon 922, Kontron Ltd., Germany). Guaiacol peroxidase activity (POD) was determined according to Castillo et al. (1984), superoxide dismutase activity (SOD) according to McCord and Fridovich (1969), ascorbate peroxidase activity (APX) according to Peters et al. (1989) and glutathion reductase activity (GR) according to Castillo and Greppin (1988). All the results were expressed on a dry weight (DW) basis.

Peroxidase isozymes were determined in leaf samples of ten different saplings, each originated from a different mother tree per origin (rock and garrigue). Thin-layer isoelectric focusing was performed in Ampholine-polyacrylamide gels (Pharmacia Biotech GmbH, Uppsala, Sweden) in a 3.5–10 pH gradient (Castillo et al. 1984). Enzyme extracts (10 µl) were applied to the gel layer. Focusing was carried out at constant power (30 W) at a maximum of 1,500 V for 2 h. Isoperoxidase bands were stained with 2 mM tetramethylbenzidine and 3 mM $H_2O_2$ in Na-acetate buffer, pH 4.5. The isoperoxidase bands were scanned and analysed. The presence or absence of each band was recorded. Patterns were identified and grouped by frequencies. The degree of similarity between populations was estimated by means of the Rogers and Tanimoto Coefficient ($S_{rt}$, Sneath and Sokal 1973).

### Determination of the total antioxidant activity and ascorbic acid content

Total antioxidant activity (TAA) is related to compounds that can protect a biological system against the potentially harmful effect of processes or reactions that cause excessive oxidation. This activity was determined according to Cano et al. (1998), with some modifications. The method is based on the capacity of antioxidant substances to scavenge the oxidant radical $ABTS^+$ [2,2′-azino-bis-(3-ethyl-benzothiazoline-6-sulfonic acid)]. TAA was quantified in plant

extracts by measuring the decrease in the absorption of $ABTS^+$ caused by the addition of an aliquot of plant extract to the reaction mixture, compared to the scavenging capacity of a standard antioxidant (ascorbic acid). Leaf extraction was carried out on 0.2 g of frozen sample that were homogenized in 4 ml of extraction buffer containing 50 mM $KH_2PO_4/Na_2HPO_4$ pH 7.5 using a Mikrodismembrator (Braun Ltd., Germany). The homogenate was centrifuged at 20,000*g* for 10 min at 4°C. The supernatant was collected and used for assays of the antioxidant activity. TAA quantification assays were performed spectrophotometrically (Uvikon 922, Kontron Ltd., Germany) at 730 nm in a total volume of 1 ml at 25°C. The reaction mixture contained 2 mM ABTS, 15 µM $H_2O_2$ and 0.25 µM horseradish peroxidase in 50 mM phosphate buffer pH 7.5. When $ABTS^+$ was stabilized, an aliquot of 5 µl of plant extract was added to the reaction mixture. The decrease in absorbance was proved to be proportional to the concentration of reductant species present in the sample. Total antioxidant activity (TAA) was calculated as the amount, expressed as ascorbic acid equivalents, which produced the same antioxidant effect as the sample under study.

The determination of ascorbic acid (AsA) content was performed on the same leaf extract used previously in the TAA quantification. Two units of purified ascorbate oxidase (Sigma, USA) were added to 10 µl of leaf extract to completely oxidize the ascorbic acid contained in the sample. After 5 min, the antioxidant activity was determined again as previously described, and the difference between the latter measurement and the TAA determined before was considered to be the antioxidant capacity of ascorbic acid in the sample.

### Biomass, structural and morphological parameters

Six plants per species, treatment and origin were harvested at the end of the experiment to analyse the pattern of biomass distribution to roots, shoots and leaves. To this end, the different parts of each plant were separated and, after drying them in an oven at 70°C during 48 h until constant weight, several structural parameters were determined: height, maximum root length, stem diameter, individual leaf area (LA), specific leaf area (SLA, leaf area per leaf dry weight), total plant leaf area (TLA), total plant dry weight biomass (TB), root dry weight per plant weight ratio (RWR) and leaf weight ratio (LWR, leaf dry weight per plant dry weight).

### Plasticity index

Phenotypic plasticity for a given trait ($x$) and species was estimated from the absolute difference in the values of $x$ between the two individuals of the same species and population grown in different treatments. The relative distance plasticity index (RDPI, Valladares et al. 2006), ranging from 0 to 1, was calculated for each trait, species and population as $RDPI = \Sigma(d_{ij} \rightarrow i'j')/(x_{i'j'} + x_{ij})/n$ where $n$ is the total number of distances and j and j′ are two individuals of the same species belonging to different treatments (i and i′). RDPI was calculated both for each species as a whole and for each population separately.

### Statistical analysis

In the species model, the factor population was nested within species, since garrigue populations of each species are evolutionary independent, and a two-way ANOVA was performed with water treatment and species as fixed factors in the model (ANOVA, Tukey test, JMP 5.0, SAS Institute Inc., USA). The number of saplings used for testing the effect of each factor combination was six. Assumptions of normality and homoscedasticity were tested by Kolmogorov–Smirnov and Levene test, respectively.

## Results

### Isozyme similarity index

Electrophoretic separation of isoperoxidases yielded five different patterns (A–E) in *Q. coccifera* and seven (a–g) in *P. halepensis* with a variable number of bands (7–12, Fig. 1). Higher number of dominant patterns were observed in the rock populations, where B patterns accounted for 7 of 10 saplings in *Q. coccifera*, and "a" and "b" patterns accounted for 9 of 10 saplings in *P. halepensis*. We also found differences between origins in the isozyme population similarity index ($S_{rt}$, Table 2), since both the species exhibited a higher similarity at the rock site (0.75 and 0.84 in *Q. coccifera* and *P. halepensis*, respectively) than at the garrigue site (0.67 and 0.77 in *Q. coccifera* and *P. halepensis*, respectively). Peroxidase electrophoretic patterns also showed that populations were more similar within origins than between them (0.55 and 0.67 in *Q. coccifera* and *P. halepensis*, respectively), with *P. halepensis* always exhibiting higher similarity than *Q. coccifera*.

### Functional responses of saplings to water availability

As expected, saplings of both species from the water-stress treatment had lower predawn and midday water potentials than well-watered saplings ($P < 0.01$), (Fig. 2, appendix 1 in electronic supplementary material). Water deficit reduced stomatal conductance (gs; $P < 0.001$) and, as a consequence, both net $CO_2$ assimilation ($P < 0.001$) and

*Q. coccifera* patterns

| Bands | A | B | B | C | B | B | B | B | B | A | C | C | D | D | D | E | E | C | C | C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | - | - | + | - | - | - | - | - | - | + | + | - | - | - | + | + | + | + | + |
| 2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 3 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 4 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 5 | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + |
| 6 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 7 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 8 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | + |
| 9 | + | + | + | - | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| 10 | + | + | + | - | + | + | + | + | + | + | - | - | + | + | + | - | - | - | - | - |
| 11 | - | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + |
| 12 | - | + | + | + | + | + | + | + | + | - | + | + | - | - | - | - | - | + | + | + |
| | Rock | | | | | | | | | | Garrigue | | | | | | | | | |

*P. halepensis* patterns

| Bands | a | b | a | b | b | b | a | a | c | b | d | d | d | e | d | b | f | b | f | g |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 2 | + | + | + | + | + | + | + | + | + | + | - | - | - | - | + | - | - | - | - | + |
| 3 | + | - | + | - | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - |
| 4 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 5 | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + | - | - | - | - | - |
| 6 | + | - | + | - | - | - | + | + | + | - | + | + | + | + | + | - | + | - | + | + |
| 7 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 8 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 9 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 10 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 11 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| 12 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | Rock | | | | | | | | | | Garrigue | | | | | | | | | |

**Fig. 1** Composition of the leaf isoenzymatic peroxidase patterns of ten saplings of *Q. coccifera* (A–E) and *P. halepensis* (a–g) from two different sites (rock and garrigue). Within each isoenzymatic pattern, the presence (+) or absence (−) of each band is indicated

**Table 2** Population similarity index (Sr) in individuals of *Q. coccifera* and *P. halepensis* from two different sites

| | *Q. coccifera* | *P. halepensis* |
|---|---|---|
| Rock | 0.75 | 0.84 |
| Garrigue | 0.67 | 0.77 |
| Rock–garrigue | 0.55 | 0.67 |

transpiration rates ($P < 0.01$) were lower in water stressed than in well-watered saplings in both the species. Water stress also exacerbated midday down-regulation of the photochemical efficiency (dynamic photoinhibition) reducing the quantum yield of noncyclic electron transport ($\Phi_{PSII}$), photochemical quenching (qP) and photochemical efficiency of the open reaction centers of PSII ($F_v'/F_m'$) in water-stressed saplings as compared to well-watered saplings (Fig. 3, $P < 0.01$). Dynamic photoinhibition involved an increase of thermal dissipation of excess energy because at the same time non-photochemical quenching (qN, Fig. 3) was higher in water-stressed saplings than in well-watered saplings ($P < 0.05$). However, water stress not only induced dynamic photoinhibition but also a reduction in $F_v/F_m$ (Fig. 3, $P < 0.01$). The higher stress of saplings from water-deficit treatment resulted in a loss of pigment content (chlorophyll a, chlorophyll b and total carotenoids, $P < 0.05$) and an increase of the carotenoids/chlorophyll ratio (appendix 2 in electronic supplementary material). Water stress also caused an increase of the antioxidant capacity since all antioxidant enzymes (POD, APX, SOD and GR), ascorbic acid (AsA) and total antioxidant activity (TAA) were higher in water stressed than in well-watered saplings of both *Q. coccifera* and *P. halepensis* (Fig. 4, $P < 0.001$). Finally, water stress also caused some modifications in growth, structural parameters and biomass allocation. Hence, water stressed saplings exhibited lower height, root length, stem diameter, specific leaf area (SLA), total leaf area (TLA) and leaf weight ratio (LWR) than well-watered saplings (appendix 3 in electronic supplementary material). Water-stressed saplings increased the fraction of the biomass allocated to roots as compared to well-water saplings ($P < 0.05$).

Seed origin (population) had no effect on any of the water potential, water content, gas exchange, fluorescence, pigments, growth or structural parameters studied in any of the two species. In contrast, important differences were found between the species since *P. halepensis* always showed higher (less negative) water potential and leaf water content and lower stomatal conductance, net $CO_2$ assimilation and transpiration rate than *Q. coccifera* ($P < 0.01$). *P. halepensis* avoided chronic and intense photoinhibition as evidenced by its higher midday $\Phi_{PSII}$, $F_v'/F_m'$ and qP, as compared to *Q. coccifera*, (Fig. 3, $P < 0.01$). *P. halepensis* also showed higher predawn $F_v/F_m$ values than *Q. coccifera* (Fig. 3, $P < 0.01$), indicating a higher permanent photoinhibition in the latter species. *Q. coccifera* had higher pigment content than *P. halepensis*, although the carotenoid/chlorophyll ratio was higher in *P. halepensis* (appendix 2 in electronic supplementary material), $P < 0.001$). Species also differed in their antioxidant capacity, since all the parameters studied, with the exception of superoxide dismutase (SOD), were higher in *Q. coccifera* than in *P. halepensis* (Fig. 4, $P < 0.01$). Both the species differed in biomass partitioning as well, with *Q. coccifera* allocating a higher proportion of biomass to roots and *P. halepensis* to leaves, as RWR was higher in *Q. co*ccifera and TLA and

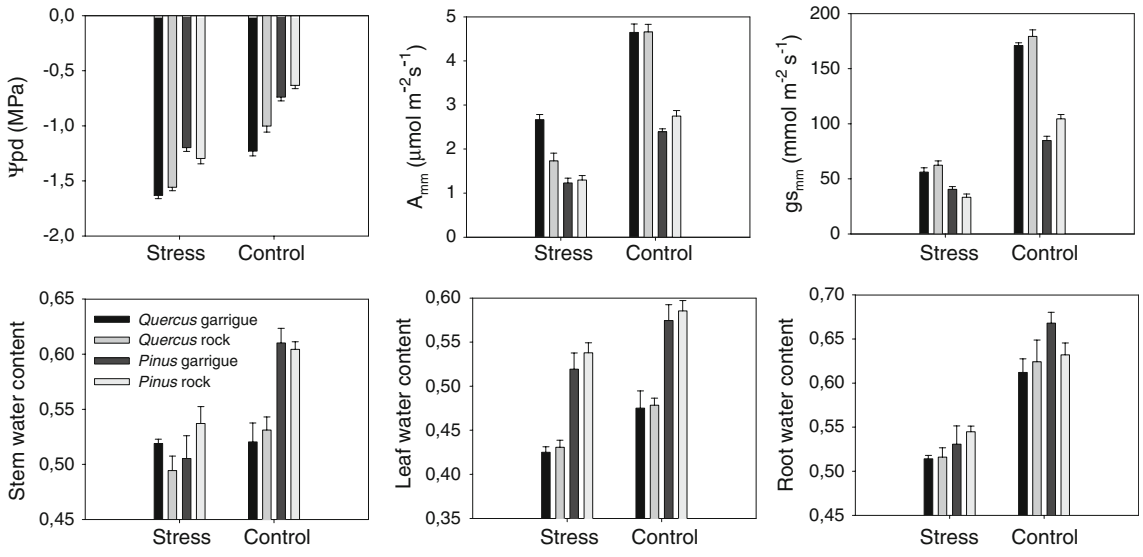


**Fig. 2** Predawn stem water potential ($\Psi_{pd}$,), midmorning net $CO_2$ assimilation ($A_{mm}$,), midmorning stomatal conductance ($gs_{mm}$,) and stem, leaf, and root water content, of *Q. coccifera* and *P. halepensis* saplings from two different provenances (garrigue and rock) and maintained under two different water treatments (control and water stress). Data are the mean ± SE ($n$ = 6–10)

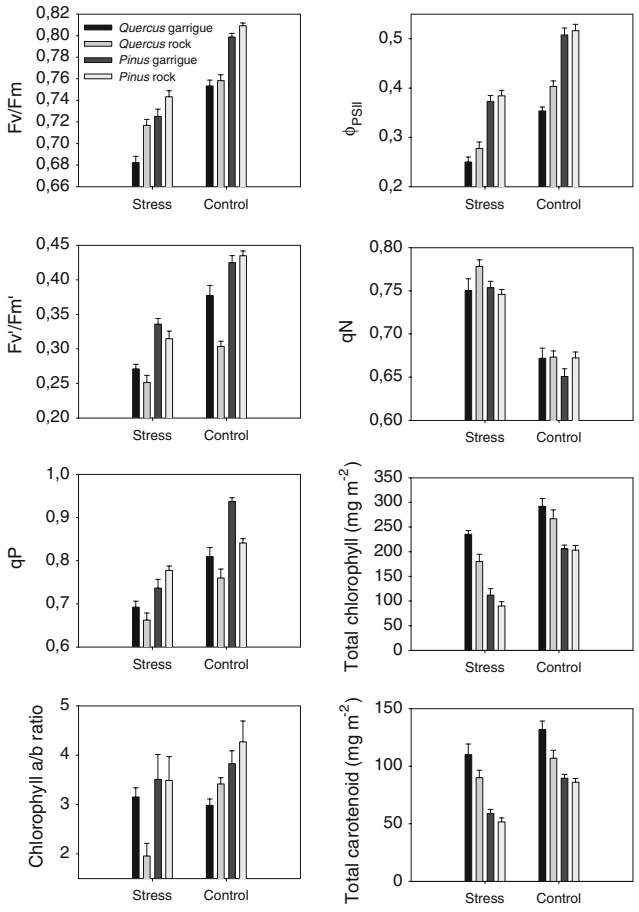


**Fig. 3** Maximum photochemical efficiency of PSII ($F_v/F_m$), quantum yield of non-cyclic electron transport ($\Phi_{PSII}$), photochemical efficiency of the open reaction centers of PSII ($F_v'/F_m'$), non-photochemical quenching (qN), photochemical quenching (qP), total chlorophyll content, chlorophyll a/b ratio and total carotenoid content of *Q. coccifera* and *P. halepensis* saplings from two different provenances (garrigue and rock) and maintained under two different water treatments (control and water stress). Data are the mean ± SE ($n$ = 6–10)

LWR were higher in *P. halepensis* ($P < 0.001$, appendix 3 in electronic supplementary material).

### Phenotypic plasticity

Most of the variability among individual plants was caused by plastic responses to water stress (75%, Table 3). Despite the small differences in the response to water availability between provenances, *P. halepensis* saplings from the garrigue site showed significantly higher RDPI values than saplings from the rock site (0.24 and 0.22, respectively, $P < 0.01$); this was not the case for *Q. coccifera* (Fig. 5; Table 3). Main plasticity differences were due to a higher plasticity in pigments, antioxidants and structural characters in the garrigue saplings of *P. halepensis* ($P < 0.01$). Both the species presented the highest plasticity in gas exchange traits and the lowest in fluorescence traits (Table 3). Mean plasticity of *P. halepensis* (0.31) was higher than that of *Q. coccifera* (0.28, $P < 0.05$). Significant differences between species were found in water relations, gas exchange, fluorescence and antioxidant traits, for which *P. halepensis* was more plastic than *Q. coccifera* ($P < 0.05$).

## Discussion

The ecotypic differentiation between the populations observed here was evidenced by the fact that both the species exhibited dominant isozyme patterns in plants from the rock populations, which were those exposed to the most adverse conditions regarding nutrient and water availabilities. The lower dominance of a few genotypes in plants from the garrigue site suggests the coexistence of several genotypes under the relatively more favorable conditions of this site. Collectively, these results suggest selection of specialized genotypes under adverse conditions. This ecotypic differentiation was also observed in phenotypic features, in agreement with a number of reports of population differentiation for a wide range of physiological and structural parameters including plastic responses to environmental factors (Balaguer et al. 2001; Abrams 1994; Gratani et al. 2003; Gonzalez and Gianoli 2004). Gratani et al. (2003) observed in *Q. ilex* saplings from different Italian provenances that saplings from more xeric provenances had higher tolerances to drought. However, in our case there were no systematic differences in the response to water stress between rock and garrigue populations, consistent with adaptive expectations in *Q. coccifera* or *P. halepensis* saplings. Greater contrast between the origins was expected for the contrasting populations of these two evergreen species, particularly after the observed differences in the isozyme patterns. In fact, such different isozyme patterns were associated with significantly different degrees of phenotypic plasticity in contrasting populations of one of the two species studied here (*Q. coccifera,* Balaguer et al. 2001).

The ability of a plant to acclimate to different environments is, in general, genetically determined (Donohue et al. 2005; Lin and Hsu 2004; Miner et al. 2005; Ronsheim and Bever 2000; Schlichting and Pigliucci 1998; Sultan 2000). It has been documented that while some species exhibit substantial capacity for acclimation, other show only a modest plastic response to environmental change (Gonzalez and Gianoli 2004; Valladares et al. 2007). The differences in acclimation capacity and phenotypic plasticity have been shown to be different also among populations of a given species (Balaguer et al. 2001; Einhorn et al. 2004; Gratani et al. 2003; Gianoli 2004; Lauteri et al. 2004; Saldaña et al. 2005; Yamashita et al. 2002, among others). Some authors have proposed that specialization in favorable environments is linked to an increased plasticity, whereas the

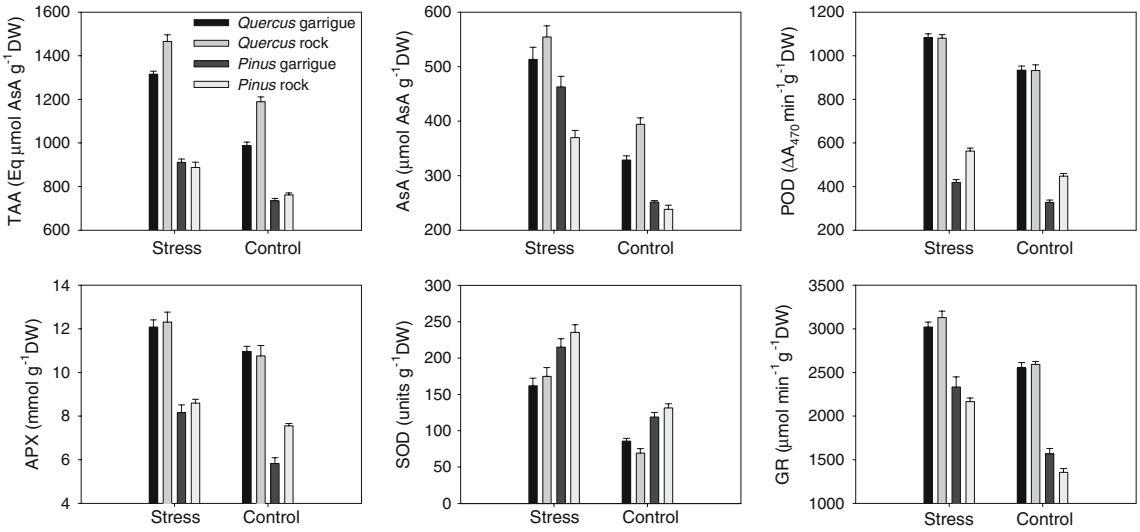


**Fig. 4** Total antioxidant activity (TAA), ascorbic acid content (AsA), guaiacol peroxidase activity (POD), ascorbate peroxidase activity (APX), superoxide dismutase activity (SOD) and glutathione reductase activity (GR) of *Q. coccifera* and *P. halepensis* saplings from two different provenances (garrigue and rock) and maintained under two different water treatments (control and water stress). Data are the mean ± SE ($n$ = 6–10)

**Table 3** Phenotypic plasticity index and percent of variation explained within origins for water relations, gas exchange, fluorescence, pigments, antioxidant and structural parameters in *Q. c*occifera and *P. halepensis* for the 36 characters determined

| | *Q. coccifera* | | | | *P. halepensis* | | | |
|---|---|---|---|---|---|---|---|---|
| | Plasticity index | | | Variation explained within origins (%) | Plasticity index | | | Variation explained within origins (%) |
| | RDPI | | Total | | RDPI | | Total | |
| | Garrigue | Rock | | | Garrigue | Rock | | |
| Water relations | $0.13^{a}$ | $0.12^{a}$ | $0.15^{ab}$ | 78–84 | $0.16^{b}$ | $0.17^{bc}$ | $0.19^{c}$ | 81–91 |
| Gas exchange | $0.35^{a}$ | $0.35^{a}$ | $0.48^{c}$ | 73 | $0.41^{b}$ | $0.43^{b}$ | $0.55^{d}$ | 74–77 |
| Fluorescence | $0.07^{a}$ | $0.11^{b}$ | $0.18^{d}$ | 40–61 | $0.14^{c}$ | $0.12^{bc}$ | $0.21^{e}$ | 59–67 |
| Pigments | $0.23^{a}$ | $0.25^{a}$ | $0.28^{b}$ | 83–90 | $0.17^{a}$ | $0.12^{a}$ | $0.23^{b}$ | 73–86 |
| Antioxidants | $0.15^{a}$ | $0.16^{a}$ | $0.20^{b}$ | 74–78 | $0.24^{c}$ | $0.17^{a}$ | $0.26^{c}$ | 65–61 |
| Structural | $0.23^{a}$ | $0.21^{a}$ | $0.29^{b}$ | 73–77 | $0.22^{a}$ | $0.19^{a}$ | $0.27^{b}$ | 69–82 |
| Mean | $0.20^{a}$ | $0.21^{a}$ | $0.28^{c}$ | 73–75 | $0.24^{b}$ | $0.22^{a}$ | $0.31^{d}$ | 72–79 |

Different superscript in each row indicates significant differences (ANOVA, $P < 0.05$)

reverse can be expected for specialization in less favorable environments (Balaguer et al. 2001; Lortie and Aarssen 1996; Valladares et al. 2000b, 2007). Our results in *P. halepensis* support this notion, since saplings from the garrigue site were more plastic, and also agree with the idea that ecotypic differentiation involved the coexistence of a higher number of genotypes under favorable conditions (Balaguer et al. 2001). However, the lack of interpopulation differences in *Q. coccifera,* which is consistent with other studies (e.g., Sultan 1996), suggests that phenotypic plasticity can hide selective differences among genotypes, maintaining genetic variation largely unavailable to selection.

Most of the water relations, gas exchange, fluorescence, pigments, antioxidants and structural characters were plastic in response to water availability in *Q. coccifera* and *P. halepensis*, although phenotypic plasticity was relatively low as compared to other plants (e.g., evergreen tropical species studied in Valladares et al. 2000b). This low-phenotypic plasticity found here in *Q. coccifera* and *P. halepensis* is in accordance with previous studies on Mediterranean species (Balaguer et al. 2001; Valladares et al. 2000a, 2002b) and also support the idea that specialization to adverse environments is linked to phenotypic stability and a conservative resource-use strategy, even when resources are temporarily abundant, in order to avoid the production of a plant too large or structures too expensive to be sustained once conditions deteriorate. Aerts (1995) also suggested that growth characteristics of evergreens lead to a low responsiveness to environmental changes. Nevertheless, the responsiveness of *Q. coccifera* and *P. halepensis*

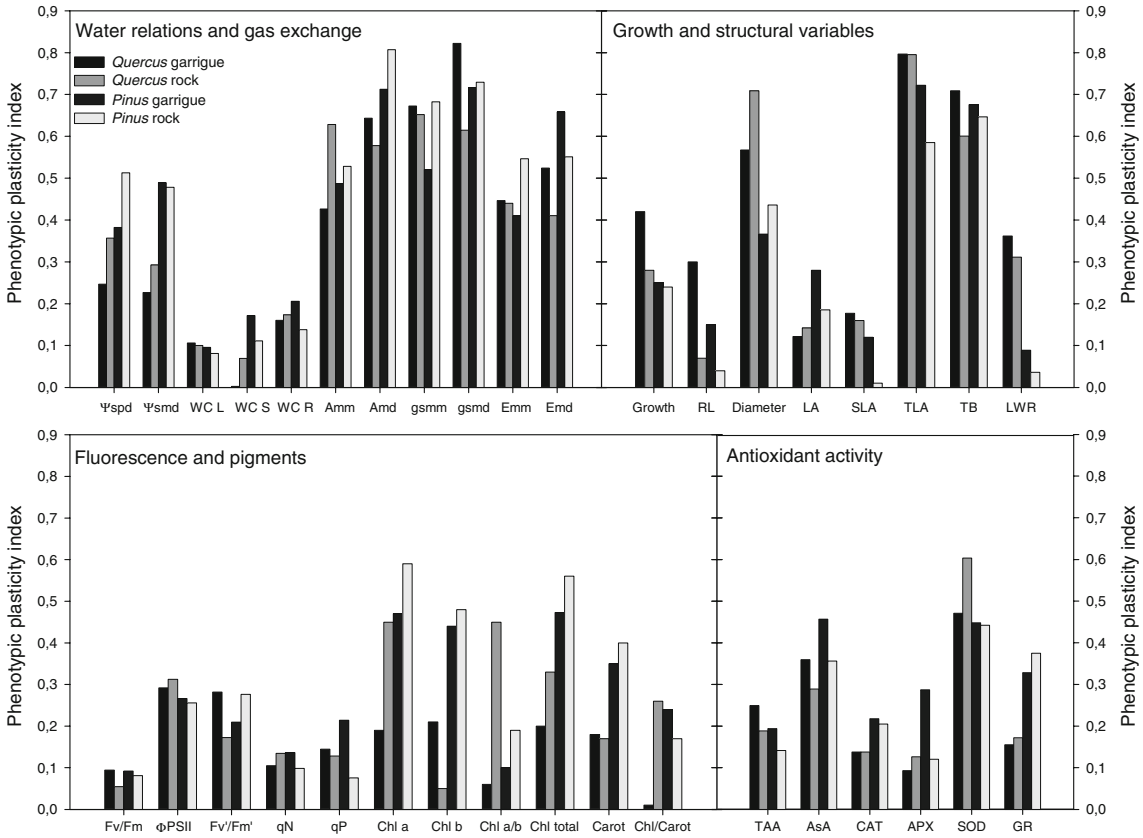


**Fig. 5** Relative distance plasticity index (RDPI, Valladares et al. 2006) for parameters that responded significantly to water stress in saplings of *Quercus coccifera* and *Pinus halepensis* originated from contrasted populations (garrigue and rock). Variables were grouped under "*Water relations and gas exchange*", "*Growth and structural variables*", "*Fluorescence and pigments*" and "*Antioxidant activity*"

to water availability, which was similar to that found in other studies of Mediterranean oaks in response to light and nutrients (Balaguer et al. 2001; Castro-Diez et al. 2006; Gratani et al. 2003; Valladares et al. 2000a), was remarkably lower than that of evergreen tropical rainforest shrubs (Valladares et al. 2000b), revealing large differences in responsiveness within evergreen woody plants from different habitats.

The differences found in water potential, stomatal conductance and net $CO_2$ assimilation (always higher in *Pinus halepensis* than in *Quercus coccifera*), can be associated to the different water use strategy of each species since *P. halepensis* behaves as a drought-avoider and *Q. coccifera* as a drought-tolerant species (Martínez-Ferri et al. 2000). *Q. coccifera* and *P. halepensis* are also known to have a different degree of dynamic photoinhibition, with *P. halepensis* avoiding photoinhibition and *Q. coccifera* being more tolerant to it (Martínez-Ferri et al. 2000). Although species differences in plasticity were modest, we expected both the species to differ in their phenotypic plasticity on the basis of their different functional strategy and habitat preferences. Specifically, we expected that *P. halepensis*, the species with the widest ecological distribution, was more plastic than *Q. coccifera,* which was confirmed by our results (Table 3). However, the relatively similar mean plasticity of both the species suggests that even ecologically and functionally distinct species converge in their capacities to respond to environmental heterogeneity by means of plastic phenotypic adjustments (Robinson and Rorison 1988). The species did differ, though, in their plasticity for key individual traits: *P. halepensis* exhibited higher plasticity for water relations, pigment and antioxidant characters, whereas *Q. coccifera* showed higher plasticity for structural characters. These results, together with previous findings (e.g., Balaguer et al. 2001; Castro-Díez et al. 2006; Navas and Garnier 2002; Valladares et al. 2002b), reveal that selection for plasticity is not only different under different levels of adversity but also that it strongly depends on the trait or suite of traits examined.

The species studied here presented high plasticity for gas exchange variables in response to water stress, revealing a noticeable stomatal control, which in turn determines net $CO_2$ assimilation and transpiration. The low plasticity detected for fluorescence characters indicates that both the species maintain their photochemical efficiency by adjustments of other physiological characters, such as modifications of

either photorespiration, pigment content or antioxidant capacity, especially in *P. halepensis*. This higher plasticity of *P. halepensis* for pigment and antioxidant characters could be related to the photoinhibition-avoiding strategy found in this species, since it would be a mechanism to reduce light absorption by PSII, dissipate excess energy and prevent overexcitation (Kyparissis et al. 2000, Martínez-Ferri et al. 2000).

Phenotypic plasticity in response to water explained over 75% of the total variability among individual plants. Thus, although some ecotypic divergence between the rock and the garrigue populations were found, saplings were plastic enough to blur this ecotypic divergence (Fig. 1; Table 2). It has been argued that the environmental unpredictability such as that characterizing most Mediterranean ecosystems would favor a reduced phenotypic plasticity and a reduced expression of the genetic variability (Kawecki 2000; Valladares et al. 2002a, 2007). This phenotypic canalization can be coupled to a genetic canalization where certain genotypes become dominant under adverse conditions. However, plasticity, even if modest as observed here, can blur population differences in functional traits, blurring in turn ecotypic divergence. Nevertheless, this conclusion must be taken as a working hypothesis for future research due to the low number of individuals sampled per population.

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