

Highlights

- Genetic variation of *Quercus ilex* promotes differences in drought tolerance among ecotypes
- *Q. ilex* ecotypes, grown under identical environmental conditions, exhibit significant variation in morphological and physiological traits.
- Variables related to seasonal precipitations and evapotranspiration showed the higher correlation with ecotypes response.

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1 **Growth and physiological sapling responses of eleven *Quercus ilex* ecotypes**
2 **under identical environmental conditions**

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16 **Abstract**

17 Studies with holm oak indicate that genetic variation may result in substantial differences in
18 drought tolerance among its ecotypes. However, few trials have studied this variation under
19 common environmental conditions. This study aimed to assess physiological and
20 morphological responses of holm oak saplings for 11 ecotypes that represent a longitudinal
21 transect across south-central Spain (Andalusia). Drought resistance was assessed by
22 measuring growth, xylem water potential, chlorophyll fluorescence, and photosynthesis.
23 Possible relationships among morphological and physiological responses across ecotypes
24 were determined using Pearson product-moment correlations and multiple linear regressions.
25 The response variables were used in multivariate analyses including discriminant function
26 analysis, principal component and cluster analyses. Last, we used sparse Partial Least Squares
27 regression (sPLS) to analyse the relationships between the morpho-physiological responses
28 and biophysical parameters of the parent locations. Our results indicate that *Q. ilex* ecotypes
29 growing in a common garden setting exhibited substantial variation in morphological and
30 physiological traits. At the end of the growth trial (65 months post-planting), basal diameter,
31 leaf area, and midday water potential were higher in *Q. ilex* ecotypes from western sites
32 compared to eastern sites across Andalusia. PCA and clustering revealed clear morphological
33 and physiological differentiation in response to gradients of geographical and ecological
34 variation in ecotype origin. Variables that were related to the water regime of the ecotypes,
35 such as seasonal precipitation and evapotranspiration, showed stronger correlations with
36 ecotype responses. Consequently, eastern ecotypes were more likely to spread in response to
37 projected increases in temperatures and declines in summer precipitation; however, western
38 ecotypes would likely decrease in response to hotter and drier summers.

39 **Key words:** Drought tolerance, provenance, chlorophyll fluorescence, water potential, holm
40 oak, afforestation

42 **Introduction**

43 The holly or holm oak (*Quercus ilex*) grows in parts of southern Europe, Asia Minor, and
44 North Africa, surrounding the Mediterranean Sea. Forests of this evergreen species grow on a
45 range of substrates and in semi-arid and sub-humid climates of the Mediterranean region,
46 although their optimal development occurs under annual rainfall regimes of 350-700 mm and
47 mean minimum temperatures ranging between -2 and 10 °C (Barbero et al., 1992). Total
48 forest cover is estimated to be about 3.9 million hectares, and has great ecological importance
49 in the southern Mediterranean Basin (García-Nogales et al., 2016). Holm oak populations are
50 often fragmented, resulting in prolonged isolation and complex geographic patterns of genetic
51 variation (Guzmán et al., 2015). Mediterranean savanna-like agrosylvopastoral systems
52 (*dehesas* in Spain, *montados* in Portugal and the *terroir* of France) are managed and highly
53 manipulated systems (Joffre et al. 1999), which may help explain the observed genetic
54 variability from location to location.

55 *Quercus ilex* L. subsp. *ballota* (Desf.) Samp. is the subspecies of holm oak that is native to
56 Spain. It is one of the most widely planted trees in reforestation and afforestation sites,
57 especially within the southern part of the country. These areas cover more than 82 755 ha in
58 Andalusia, particularly in abandoned agricultural lands. Holm oak has been selected for
59 afforestation because of its high drought tolerance (Barbeta & Peñuelas, 2016) and plasticity
60 of response to varying edaphic conditions (Laureano et al., 2016). In southern Spain, holm
61 oak forests occur over a wide range of rainfall regimes, i.e., from drought-affected marginal
62 lands with annual rainfall as low as 260 mm to wet climates with annual rainfall over 1000
63 mm. These populations may be particularly suitable for forest conservation and afforestation
64 projects that place a premium on stress tolerance over growth rate. Although several studies
65 have investigated the relevant seedling traits in nurseries prior to out-planting (Villar-

66 Salvador et al., 2004), physiological and morphological analysis of these populations and
67 their responses to water deficits and identification of the traits conferring drought resistance
68 are required (Costa-Saura et al., 2016), once the plants (saplings) have become established in
69 the field after several years.

70 Adaptation of species to geographic variation in the environment often depends upon genetic
71 variation among seed sources. Genetic inventories using biochemical and DNA markers have
72 demonstrated that genetic variability is geographically structured. Variation in holm oak is
73 greatest in France (Lumaret et al., 2002; Vitelli et al., 2017) and Spain (Guzmán et al., 2015;
74 García-Nogales et al., 2016), most likely due to the genetic structure of populations and their
75 historical management. Genetic studies, together with ecological information, have been used
76 to define seed orchards of holm oak in several countries. In general, seed sources from drier,
77 inner portions of this species' range show greater pre-adaptation to drought than populations
78 originating close to the coast (Matías et al., 2010). Studies with holm oak indicate that genetic
79 variation may promote significant differences in drought tolerance among ecotypes (Peguero-
80 Pina et al., 2014). However, the production of *Q. ilex* nursery stock suffers from a low degree
81 of genetic variability, which may limit the success of more drought tolerant *Q. ilex* ecotypes
82 in restoration and reforestation programs.

83 *Quercus ilex* occupies habitats that receive low amounts of precipitation, but elevated levels
84 of annual radiation (Quero et al. 2006); under planting stress, seedlings may undergo
85 morphological and physiological changes, such as leaf morphology and transpiration
86 reduction, by decreasing the area of exposed leaf surface. Variation in stem and leaf
87 morphology, seedling physiology, phenological stages, and growth have been reported among
88 holm oak populations that were raised from different seed sources (Gratani et al., 2003;
89 Gimeno et al., 2009; Peguero-Pina et al., 2014), but relatively little information exists

90 concerning genetic variation in the major components of drought resistance in this species.

91 There is increasing interest in using species' traits to predict ecotype responses to
92 environmental change (Niinemets, 2015, Cavender-Bares et al., 2016). This approach has
93 been used in studies of *Quercus ilex* responses to drought (Fusaro et al., 2017), climate
94 gradients (García-Nogales et al., 2016; Peguero-Pina et al., 2014) and other forms of
95 disturbance (Chiatante et al., 2015). It has been proposed that attention be focused on plant
96 organs such as leaves to facilitate functional comparisons of plants, since leaves are the most
97 well-studied plant organs (Wright et al., 2004; Pérez-Harguindeguy et al., 2013). Leaf traits
98 are strongly correlated among populations and species. This implies that multiple traits are
99 associated with a singular trade-off in function (Wright et al., 2004).

100 Therefore, a wide range of ecophysiological responses is expected in *Q. ilex* ecotypes
101 originating from habitats of differing moisture regimes, when grown under identical field
102 conditions. In previous studies, differences in growth, leaf gas-exchange, and xylem water
103 potential were observed, suggesting that the genetic diversity of *Q. ilex* ecotypes leads to
104 differences in establishment success (Gratani et al., 2003; Pesoli et al., 2003). This differential
105 response indicates that *Q. ilex* possesses highly effective physiological plasticity and can thus
106 adapt to different environmental conditions (Gimeno et al., 2009; Peguero-Pina et al., 2014).

107 Yet, the genetic sources of *Q. ilex* plasticity have been explored to a lesser degree than those
108 of other forest species (Michaud et al., 2005; Valero-Galván et al., 2011, 2012) in long-term
109 field trials. Hence, the results lead to reliance upon particular genotypes or ecotypes.

110 Consequently, analysis of the physiological and morphological responses of *Q. ilex* ecotypes
111 under identical environmental field conditions is crucial to the characterisation of the species
112 and the selection of the more drought-tolerant genotypes (ecotypes) among the provenances.

113 We hypothesised that the more drought-resistant *Q. ilex* ecotypes might be less susceptible to

114 water stress, possibly due to lower rates of photosynthesis and growth; i.e. a more
115 conservative resource-use strategy (sensu Valladares et al., 2000).

116 The aim of this study was to assess how morphological and physiological traits of 11 *Quercus*
117 *ilex* ecotypes affect their performance under identical environmental conditions. Therefore, in
118 this study we proposed and tested three non-exclusive hypotheses: i) Are there differences in
119 morphological and physiological traits among *Q. ilex* ecotypes? ii) Are *Q. ilex* populations
120 from drier habitats more drought-tolerant than those from moister ones? iii) Can we identify,
121 several years after establishment, which populations and plant traits may warrant further
122 ecotype selection for the improvement of *Q. ilex* drought tolerance for forest conservation and
123 afforestation programs in southern Spain? We expect that ecotypes responses vary according
124 to the environmental conditions of populations' origin.

125 **Materials and methods**

126 *Plant material and growth conditions*

127 Since 2009, the University of Córdoba has hosted a collection of 11 different ecotypes of *Q.*
128 *ilex*. Each one consists of a group of plants grown from acorns that were collected from
129 controlled seed sources provided by the Andalusia Forest Department (southern Spain, Table
130 1, Figure S1 Supplementary material). The ecotypes that were included in this study were
131 selected according to their potential use in afforestation under drought conditions, covering a
132 wide range of habitats in southern Spain (region of Andalusia). To establish this collection,
133 one-year-old seedlings were grown with standard nursery practices (300-cm³ containers and
134 peat-vermiculite [3:1 v:v] as substrate). Average seedling height and basal diameter, measured
135 just before planting, were 14.66 ± 0.81 cm and 3.45 ± 0.31 mm, respectively (means and
136 standard errors, $N=50$), with no significant differences according to the origin of the acorns.

137 For the purposes of this study, we assumed that nursery cultivation and its environmental
138 effects during subsequent growth were minimal.

139 Seedlings were planted in a common garden at the experimental farm of Hinojosa del Duque-
140 Andalusian Institute for Research and Training in Agriculture, Fisheries, Food and Ecological
141 Products (IFAPA, Córdoba, Spain, 38°29'46"N, 5°6'55"W, 543 masl) (Figure 1). This site has
142 an average annual rainfall of 459.4 mm and a dry thermo-Mediterranean climate, with hot and
143 dry summers and warm winters (Meteorological Station of IFAPA Hinojosa del Duque;
144 <https://www.juntadeandalucia.es/agriculturaypesca/ifapa/ria>). There are 120-150 biologically
145 dry days, according to the xerothermic index of Gaussen (Emberger, 1963). The site was
146 cropped periodically thereafter and left fallow during the 2008-2009 growing seasons,
147 supporting a mixture of native and introduced herbaceous species that are associated with
148 agricultural bottomlands. The plantation was established in February 2009. The planting area
149 was sub-soiled to a depth of more than 60 cm using a ripper with a single tine, and soil clods
150 were broken up using a spring harrow and culta-mulcher to provide a more level surface for
151 planting. The planting was done by hand in a rectangular plot (150 x 75 m), following a
152 systematic spatial pattern of distribution for each ecotype, with a density of 1250 plants ha⁻¹.
153 A total of 1188 plants were distributed in the plot with a plantation grid of 4 x 2 m, grouping
154 different ecotypes (36 rows x 36 columns) in adjacent columns. Supplementary irrigation was
155 provided during the first three years to ensure seedling establishment.

156 Eleven ecotypes were considered in the trial; ecotype is a fixed effect with 10 degrees-of-
157 freedom (*df*) for analysis of variance (ANOVA). Six replicate individuals per ecotype were
158 randomly selected from across the plantation (sample size of N = 66). A series of soft and
159 hard traits were measured (Pérez-Harguindeguy et al., 2013). Among the soft traits, we
160 included anatomical-morphological features of plants (i.e. leaf area, leaf thickness and

161 specific leaf area), and among the “hard” traits, we included physiological attributes (i.e.
162 midday water potential, photosynthetic assimilation, stomatal conductance, transpiration,
163 chlorophyll fluorescence, and concentration of photosynthetic pigments).

164 *Leaf morphology*

165 In March 2009, at the time of planting, all transplanted *Q. ilex* seedlings were tagged. In
166 August 2014, 65 months after planting (average survival = 61 %), average seedling size
167 (height and basal diameter) and leaf morphological features (Table 2) were measured
168 simultaneously. To make the measurements comparable, leaves in the same position were
169 chosen and six replicate plants from each family were analysed. Leaf area (LA, cm²) was
170 measured with Image-Pro Plus software (Media Cybernetics, Inc, Rockville, MD, USA). A
171 piece of the blade was then cut, avoiding the main leaf veins, and leaf thickness (LT_h, mm)
172 was measured with an adapted electronic Calliper. The leaves were oven-dried at 80 °C for
173 three days and their specific leaf area (SLA, cm² g⁻¹) was calculated by dividing their LA by
174 their dry masses.

175 *Field xylem water potential and gas-exchange measurements.*

176 The midday (Ψ_m) leaf water potential was measured in a 2-h window around solar noon
177 (11:20-13:20 h UTC, 13:20-15:20 h CET), using a pressure chamber (SKPM 1400, Skye
178 Instruments) (Scholander et al., 1965). Measurements of field xylem-water potential were
179 taken for six plants of each family (a total of 66 seedlings) on 28 and 29 August 2014. Mean
180 (plus standard error) climatic values during the measurement periods were 34.5 ± 0.8 °C, 11.5
181 ± 2.9 % RH, 1.1 ± 0.1 km h⁻¹ wind speed, 5.27 ± 0.35 mm day⁻¹ evapotranspiration (ET_o),
182 and 23.7 ± 0.2 MJ m⁻² total solar irradiance (TSI). In combination with the water potential
183 measurements, the photosynthetic rate (A , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), stomatal conductance (G_s , mol
184 H₂O m⁻² s⁻¹), and transpiration rate (T_r , mmol H₂O m⁻² s⁻¹) were measured on fully expanded

185 sun leaves at medium height, using a portable infrared CO₂ gas analyser (LiCor Li6400XT,
186 Li-Cor, Inc., Lincoln, NE, USA) fitted with a 6-cm² leaf cuvette. The measurement conditions
187 were 390 ± 1.7 ppm CO₂, flow of 300 ± 1.2 cm³ min⁻¹, and PPFD > 1000 μmol (photons) m⁻²
188 s⁻¹; this value was enough to light-saturate this species (Quero et al., 2006). The
189 measurements were taken on 28-29 August 2014 during the morning (10:00–12:00, CET),
190 when photosynthetic rates are at their maximum.

191 *Chlorophyll fluorescence measurements*

192 Instantaneous chlorophyll fluorescence yield (F_t) of light-adapted plants and quantum yield of
193 photosystem II (QY) were measured with a portable fluorometer (FluorPen FP100, Photon
194 Systems Instruments, Drasov, Czech Republic). The measurements were recorded on three
195 south-facing leaves on six seedlings per family, at field temperature around solar noon (13:00-
196 15:00 h CET). To calculate F_t, leaves exposed to sunlight were saturated for 3 s with actinic
197 light pulses (1000 μmol (photons) m⁻² s⁻¹) to reduce all PSII reaction centres, to obtain
198 maximum fluorescence (F_m[']). The minimal fluorescence (F_o[']) was measured by applying far-
199 red light. For QY measurement, leaves were exposed to a single saturating light pulse (3000
200 μmol (photons) m⁻² s⁻¹) and the variable fluorescence (F_v[']=F_m[']-F_o[']) was determined (Orekhov
201 et al., 2015).

202 *Photosynthetic pigment contents*

203 Concentration of photosynthetic pigments in leaves was estimated using UV-Vis
204 spectrophotometry. A total of 12 leaves for each plant (6 plants per ecotype), which were
205 located on the south side of the plants and well illuminated, were collected and immediately
206 frozen in liquid nitrogen. For each leaf, two disks of 0.5 cm² were excised, avoiding leaf
207 veins, and for the mixed pool of 24 disks per plant, three technical replicates of 1 cm² were
208 ground at -20 °C; pigments were extracted in 2 ml of chilled 100 % acetone in the presence of

209 25 mg of sodium ascorbate (Abadía and Abadía, 1993). Afterwards, the absorbance of each
210 replicate was measured at 661.6, 644.8, and 470 nm using an EVOLUTION 201 UV-Vis
211 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The concentrations
212 of photosynthetic pigments in the solution were calculated using the equations described by
213 Lichtenthaler (1996). Finally, the contents of total chlorophyll ($\text{Chl}_a + \text{Chl}_b$, $\mu\text{g cm}^{-2}$),
214 chlorophyll ratio ($\text{Chl}_R = \text{Chl}_a/\text{Chl}_b$), and carotenoids (Ca , $\mu\text{g cm}^{-2}$) in the leaves were
215 calculated, adjusting for the final volume of extraction solvent.

216 *Statistical analysis*

217 Prior to statistical analysis, we examined all variables for normality using the Kolmogorov-
218 Smirnov test (with Lilliefors correction). Homoscedasticity was analysed by the Levene test
219 of variance ($P > 0.05$). When the variables were not normal, different transformations were
220 applied. Water potential responses did not follow a normal distribution, despite the
221 application of several transformations. The results in the tables are shown as means with their
222 standard errors for the untransformed variables. The morphological and physiological
223 variables were analysed 65 months after planting with one-way ANOVA, and the Kruskal-
224 Wallis test for variables with a non-normal distribution. When a global difference among the
225 provenances was detected, means were separated by Scheffe's multiple range test for unequal
226 sample sizes ($P = 0.05$) for normal and homoscedastic variables and by Nemenyi test (equal
227 replicates per group) for ranked variables (Sokal and Rohlf, 1995). Differences among
228 treatments were considered significant at a significance level of $P = 0.05$.

229 Possible relationships among the morphological and physiological responses across genotypes
230 were determined using Pearson's correlation coefficients (r) and non-parametric Spearman's
231 rank correlation (r_s) at a significance level of 5% ($P \leq 0.05$). The *Q. ilex* ecotypes are not

232 distributed randomly; they exhibit a longitudinally dependent distribution(see Table 1 and
233 Fig. S2). The response of each trait was regressed on longitude and elevation.
234 The response-associated variables were then used in a multivariate and discriminant analysis.
235 All variables satisfying conditions of normality, homoscedasticity and bivariate correlations
236 greater than 0.3 ($P < 0.05$), except for those exhibiting collinearity, were included in the PCA
237 to select the most representative ones, according to Sleighter et al. (2010). Selected variables
238 were identified by an optimal PCA solution that included Bartlett's sphericity test, a KMO
239 (Kaiser-Meyer-Olkin) test, communalities, correlations with principal components, and
240 maximum explained variance (Martínez-González et al., 2006). With variables accounting for
241 the maximum fraction of variance in the original dataset, families were subjected
242 agglomerative hierarchical cluster analysis (unsupervised clustering, Ward's method) based
243 upon squared Euclidean distance. Clustering included all selected variables (Table 6) plus
244 water potential. The original dataset was initially divided into k blocks (each observation as a
245 separate cluster), which were merged to form successively larger and fewer clusters. At each
246 iteration, squared Euclidean distance was calculated between the resulting clusters. The abrupt
247 change (i.e. the maximum slope derivative) in the sequential difference in squared Euclidean
248 distance (ΔSED) plotted against iteration step provided the stopping criterion for determining
249 optimal cluster size and number (Supplementary material, Figure S4). We tested the
250 separation of the groups that were identified in the cluster analysis using discriminant
251 function analysis (DFA), by quantifying the relationship between the resulting cluster
252 classification (categorical variable) and the set of response variables (Tabachnick and Fidell,
253 2006). Correlations between CDF scores and response variables provided information that
254 identified the most relevant variables contributing to cluster separation. The ecotypes were

255 represented in a two-dimensional ordination using CDF1 and CDF2 scores to detect the
256 presence of gradients in their respective variables.

257 Finally, we used sparse partial least-squares regression (sPLS) (Chun and Keleş, 2010, Hair et
258 al., 2012, Chung et al., 2012) to analyse the relationships between the morpho-physiological
259 response variables and the biophysical parameters of the parent location of each group
260 (edaphic, topographic, and climatic parameters, Table S1 Supplementary material). We chose
261 this approach because, even with many parameters (as are required for estimating moderating
262 effects), PLS supports sample sizes that are smaller than covariance-based structural equation
263 modelling techniques (Hair et al., 2012). We used Partial Least Squares (PLSs) regression to
264 reduce the problems of multicollinearity and the “curse of dimensionality” (sensu Bellman,
265 1957). Under the principle of sparsity (Chun and Keleş, 2010), a subset of the predictive
266 variables was identified that were assumed to drive the underlying process, thereby avoiding
267 the noise that would be incurred by inclusion of irrelevant variables in the PLS regression
268 through direction vectors.

269 The soft thresholding parameter and number of latent variables were established by cross-
270 validation for all the direction vectors, giving $\eta=0.5$ and $K=3$, respectively. Because PLS is
271 based on a non-parametric estimation procedure, we applied bootstrapping with 5000
272 resamples for the significance tests. The environmental variables that were used in the sPLS
273 as the predictor matrix ($n = 63$) included climatic ($n = 44$), topographic ($n = 8$) and edaphic (n
274 $= 11$) variables (Table S1) that were obtained from the Andalusian Environmental
275 Information Network (REDIAM). The climatic variables were averaged for the period 1971
276 to 2000. The climatic variables were averaged for the period 1971 – 2000. Response matrix
277 was composed of ecotype means ($n = 11$) for the six variables that were selected using PCA,
278 together with water potential (Ψ_m). Point-biserial correlations (r_{pb}), together with product-

279 moment correlations between all variables involved in PLS analysis (Table 1 and Table S3)
280 and the projected matrices, were represented in a Correlation Circle to evaluate the
281 association between ecotypes (defined by the response variables) and single site
282 characteristics (the predictor matrix). Partial Least Squares regression predictor matrix scores
283 were represented as a biplot, and were compared with DFA results to evaluate relationships
284 between origin site factors with the ecotype groupings.

285 All statistical analyses were performed using R version 3.4.0 (R Development Core Team,
286 2012).

287 **Results**

288 *Morphological and physiological responses*

289 Means and results of univariate ANOVA for all morphological and physiological responses
290 that were measured 65 months after planting are summarised in Table 3. Sapling height was
291 greatest (significantly so) in the Pozoblanco (Poz) ecotype (149.0 cm) and least in ecotypes
292 Almería (A) (87.8 cm) and Pozo-Alcón (Po) (89.0 cm) ($F_{10,55} = 3.04$, $P = 0.04$). A similar
293 response was observed for collar diameter, the values being lowest for the same ecotypes, 'A'
294 and 'Po' (13.4 mm and 14.0 mm, respectively), and highest for ecotypes Puerto Serrano (Ca2)
295 and Poz (28.6 mm and 27.2 mm, respectively, $F_{10,55} = 6.253$, $P < 0.001$). Leaf features
296 showed less sensitivity to ecotype origin. Significant differences were observed only for leaf
297 area ($F_{10,55} = 2.98$, $P = 0.005$), with the greatest value for the 'Poz' ecotype (4.1 cm²). Further,
298 leaf thickness ($F_{10,55} = 1.48$, $P = 0.174$) and SLA ($F_{10,55} = 0.69$, $P = 0.721$) did not differ
299 significantly among ecotypes.

300 The mean photosynthetic rate was highest (19.3 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) in ecotype A, but declined
301 to 7.2 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for the Po ecotype, with significant differences among ecotypes ($F_{10,$
302 $55 = 3.33$, $P = 0.002$); there was a similar response for stomatal conductance ($F_{10, 55} = 5.09$, P

303 ≤ 0.001) and transpiration ($F_{10-55} = 2.96, P = 0.005$) (Table 4). Ecotype had a stronger effect
304 on sapling water potential ($H_{10} = 34.82, P < 0.001$), ecotypes A (-2.3 MPa) and G (-2.5 MPa)
305 showing the highest values and Po (-4.2 MPa), H1, H2 and S1 (-4.1 Mpa) the lowest (Table
306 4).

307 During this same period, the fluorescence variables did not differ significantly ($F_t, F_{10,55} =$
308 $1.86, P = 0.074$; QY, $F_{10, 55} = 0.87, P = 0.567$) (Table 4). However, the pigment
309 concentrations showed significant differences among the ecotypes (Table 4). The
310 Benamahoma (Ca1) ecotype had the greatest concentrations of Chl_b and α & β carotenes (7.3
311 $\mu\text{g cm}^{-2}$ and $8.9 \mu\text{g cm}^{-2}$, respectively) and Segura (S) the lowest ($1.9 \mu\text{g cm}^{-2}$ and $3.5 \mu\text{g cm}^{-2}$,
312 respectively). Ecotype also had a strong effect on the chlorophyll ratio (Chl_R) ($F_{10, 55} = 5.47, P$
313 < 0.001) (Table 4).

314 Therefore, it is possible to use growth (height and collar diameter) and physiological variables
315 (A, Gs, T, Ψ_m , and pigments) to separate the sapling response groups. However, this
316 univariate approach does not account for the correlation among the variables.

317 *Correlation among response variables*

318 The correlation analysis of the physiological and morphological variables provides an
319 indication of how much each variable can contribute to the analysis (Table 5). In general, the
320 physiological variables were not significantly correlated with the morphological ones, except
321 in the case of QY and F_t . The gas-exchange and photosynthesis variables (A, Gs, and Tr) were
322 strongly intercorrelated ($P < 0.01$), as were those of the photosynthetic pigments group (Chl_a,
323 Chl_b, and Ca, $P < 0.01$). The strongest correlations ($P < 0.01$) for Ψ_m were with A ($r = 0.6$),
324 Gs ($r = 0.6$), Tr ($r = 0.6$), F_t ($r = 0.4$), and Chl_R ($r = -0.4$). Additionally, there was a significant
325 correlation between the fluorescence variables and the morphological and physiological
326 variables (F_t vs LA, $r = 0.2$; F_t vs Gs, $r = 0.3$, F_t vs Tr, $r = 0.3$; QY vs TH, $r = 0.3$; QY vs

327 SLA, $r = 0.3$; QY vs A, $r = 0.5$; QY vs Gs, $r = 0.4$; QY vs Tr, $r = 0.4$). Leaf area (LA) was
328 significantly correlated with the Chl_R, while the correlation with each pigment concentration
329 was not significant.

330 The effects of latitude, longitude, and elevation were evaluated for all ecotypes that were
331 included in the study (data not shown). The relationships with longitude and elevation were
332 significant in every case. There was a strong inverse correlation between these two variables
333 ($r = -0.761$; $P < 0.01$), a result of the distribution of selected stands in Andalusia; more
334 westerly stands were located at lower elevations. Therefore, for comparison with the other
335 data sets generated or used in this study, we focus only on longitude (see Figure S2,
336 Supplementary material, for elevation data). Mean basal diameter, LA, and Ψ_m estimates were
337 significantly higher in *Q. ilex* ecotypes that were located at increasing longitude, compared
338 with those of ecotypes that were located at decreasing longitude ($P < 0.001$; Figure 2).
339 However, the Gs, T_r, and Chl values showed the inverse response to longitude ($P < 0.001$;
340 Figure 2).

341 *Clustering and classification analysis*

342 Based upon the optimal PCA solution, the selection of the six most relevant variables was
343 made following analysis of Pearson correlations, communalities, and the structure matrix
344 (Table 6). PCA of the individual cases revealed the presence of a gradient in the ecotype
345 variables. For these variables, five natural groups were identified from the abrupt change in
346 sums-of-squares error (Δ SSE) plotted against the sequence of agglomeration iterations during
347 the hierarchical cluster analysis (HCA) of all individual cases (Supplementary material,
348 Figures S3 and S4). A first division distinguished a group composed mostly of individuals
349 belonging to western ecotypes (clusters 3, 4, and 5, squared Euclidean distance $D = 480$),
350 although it also included three individuals of eastern origin that were grouped in an isolated

351 cluster (cluster 4, $D = 273.6$). At the other geographic extreme, the two corresponding groups
352 that were comprised of eastern ecotypes were separated (clusters 1 and 2, $D = 313.7$), one
353 cluster being formed by ecotypes Almería (A) and Granada (G) (group 1) and the other by
354 Valdepeñas (P) and Segura (S) (cluster 2, $D = 242.3$).

355 When clustering was carried out for the family average data (Figure 2), the classification
356 fitted the previous analysis well. Eastern ecotypes (clusters 1 and 4, A, G, S, and P) were
357 clearly separated from the four most western ecotypes (H1, H2, S1 Ca1, Ca2, and Poz,
358 clusters 2 and 3); also, one transitional group was identified, corresponding to a
359 geographically intermediate ecotype, Po (cluster 5). The large differences between the eastern
360 ecotypes (clusters 1-4) and the rest ($D = 70.0$) are noteworthy; the distances were almost
361 double those of the next clustering step, between the extreme western ecotypes (cluster 2) and
362 the transitional group (2-3 to 5, $D = 35.5$). The plot in the re-scaled squared Euclidean
363 distance (Figure 2) shows clearly the relative importance of these differences.

364 Finally, DFA tested the degree to which the natural groupings (obtained by clustering) could
365 be separated using the morphological and physiological measurements. Globally, the
366 classification that was implemented by DFA provided a significant fit to the data (Wilks'
367 Lambda: $F_{df_1=12, df_2=10.88}, P < 0.001$). Total variation in the data was explained by only
368 three discriminant functions (Supplementary material, Table S1). The first two functions
369 provided significant fits (CDF1: $\chi^2 = 42.94, P < 0.001$; CDF2: $\chi^2 = 22.35, P < 0.001$; CDF3:
370 $\chi^2 = 5.73, P=0.057$). The discriminant scores that were estimated for the two significant
371 functions (Figure 3) clearly distinguished the geographical groupings of the ecotypes. The
372 first two axes could be interpreted as gradients in morphological and physiological conditions.
373 CDF1 was strongly correlated with Ψ_m ($r = 0.77$), while CDF2 was correlated with Chl_R ($r =$
374 0.78) and F_t ($r = 0.71$). For CDF3, QY was the principal source of variation ($r = 0.96$). Leaf

375 carotenoid concentration exhibited the lowest correlation with the CDF axes, and was
376 significant only with CDF3.

377 The two axes that were delineated by the first and second canonical discriminant functions
378 (CDF1 and CDF2) accounted respectively for 64.3 and 32.3 % of total variation. Good
379 separation of the ecotypes was achieved by CDF1, while CDF2 improved their separation.
380 The first two canonical variables were clearly able to distinguish among the five ecotype
381 groups matching the HCA groupings: Almería- Granada; Segura-Valdepeñas; Pozo Alcón;
382 Puerto Serrano-Pozoblanco; and Corteconcepción-Calaña-Almadén-Benamahoma. There was
383 little overlap among them.

384 *Environmental predictors*

385 Investigation of how specific ecotypes respond at a common garden location as a function of
386 the environmental conditions of their origin may help to predict potential impacts of different
387 growing conditions and subsequent ecotype responses. Up to 63 environmental variables were
388 used to perform sPLS regression. Following a sparsity test of the predictor matrix, the
389 ecotypes were related to 47 environmental variables (Table S2, Supplementary material),
390 given their importance in driving ecosystem processes. This analysis not only provided
391 information on how the environmental conditions at the origin may influence the distribution
392 of *Q. ilex* ecotypes, but also identified those variables that might be functionally relevant at
393 particular sites.

394 The Pearson's correlation coefficients (r) are represented in a circle of correlations (Figure 4,
395 A). Variables related to the water regime, such as seasonal precipitation, topographic wetness
396 index, and some evapotranspiration characteristics of the site of origin of the ecotypes showed
397 the same trends in the projected matrix as the Ψ_m of common garden plants, influencing the
398 distances between cases in the same direction. A similar trend was shown by F_t and LT_h ,

399 which were both strongly correlated with Ψ_m , QY, Tr, and Chl_R (all of these measurements
400 were related to photosynthetic activity), influencing the separation of the projected matrix in
401 the same way as topographic and edaphic characters, thereby defining the exposure of the
402 sites of origin. As expected, the SLA did not influence the variation among the data; finally,
403 the temperature-related variables were mostly correlated with the projected matrix in the same
404 way as the total leaf carotene content (Ca).

405 The seasonal evapotranspiration rate, excluding summer potential evapotranspiration (PET),
406 showed highly significant correlations with the photosynthetic traits and pigment variables
407 (Supplementary material, Table S2). High levels of evapotranspiration were correlated
408 negatively with Tr and Chl_R and positively with water potential, fluorescence, and carotene
409 contents, with average monthly evapotranspiration of the spring season being the most
410 strongly correlated. The mean and minimum temperatures of the coldest month were
411 correlated with pigments in the same way as evapotranspiration. High temperatures and PET
412 ratios at the site of origin of the populations led to high carotenoid contents and low Chl_R. The
413 temperature of the warmest month influenced positively the foliar thickness and the
414 fluorescence of the ecotypes. The quantum yield and SLA of the ecotypes were not correlated
415 with any biophysical variable of the sites of origin of the populations.

416 The ecotypes showed a pattern of grouping that similar to what was predicted by DFA, with
417 some interesting differences. Almería-Granada had scores that were related to low Ψ_m and
418 low topographic wetness index (TWI), water-holding capacity (WHC), and seasonal
419 precipitation. The Granada ecotype, however, was located at an intermediate position in the
420 ordination, due to the influence of climatic and biophysical variables. The Corteconcepción-
421 Almaden-Calañas and Segura-Valdepeñas ecotypes and their associations (clusters of HCA)
422 were represented in a distribution similar to that in the DFA biplot representation, being well

423 separated in the projected space. Nevertheless, three ecotypes (Pozo Alcón, Pozoblanco, and
424 Benamahoma) were represented by slightly different plot coordinates, given that they were
425 distributed randomly at the centre of the plot. The case of Puerto Serrano is of note,
426 noteworthy, since the influence of biophysical factors led to positioning of this ecotype near
427 the most western ones. Also, Benamahoma had high values of the water regime variables and
428 Ψ_m , but the influence of low-temperature-related values and high values of topographic and
429 edaphic properties led to its shift to the centre of the plot, near to the transitional ecotypes.

430 **Discussion**

431 Holm oak (*Quercus ilex* L.) is the most abundant *Quercus* tree species in the Mediterranean
432 region, covering a large ecological gradient across the western Mediterranean Basin (Gratani
433 et al., 2003; Martin-St. Paul et al., 2012). This ecological variability has led to high
434 intraspecific variation, with ecotype diversity manifested in the morphological (Ramírez-
435 Valiente et al., 2009; Valero-Galván et al., 2011), ecophysiological (Peguero-Pina et al.,
436 2014), and biochemical parameters (Valero-Galván et al., 2011; 2013) that were measured.
437 Many factors contribute to the high potential of *Q. ilex* to adapt to different environmental
438 conditions, which often depends on genetic variation among seed sources (Pesoli et al., 2003).
439 These adaptive features have probably been driven by the local environment from which the
440 ecotypes originate (Corcuera et al., 2004; Sánchez-Vilas and Retuerto, 2007), contributing to
441 a differential response to environmental conditions. Regarding drought responses, differences
442 have been observed in several controlled experiments (Sánchez-Gomez et al., 2006; Laureano
443 et al., 2008). Nevertheless, most studies of *Q. ilex* variability have been developed using a
444 limited number of populations (Varone et al., 2015), conducted under artificial controlled
445 conditions (García-Nogales et al., 2016) or using a biochemical approach (Valero-Galván et
446 al., 2011). In this study, we present the morphological and physiological responses under
447

448 natural conditions of *Q. ilex* ecotypes from seeds of different provenances that were located in
449 Andalusia, several years after planting, once the saplings were well established.

450 *Comparative morphology and physiology of Q. ilex ecotypes*

451 Differences among the *Q. ilex* ecotypes were found for diameter, height, and leaf area. Yet
452 ANOVA did not clearly differentiate ecotypes based upon their geographical origin (Table 4).
453 Ecotypes from the driest localities (Almería-A and Sierra del Pozo-Po) showed the smallest
454 diameter and height, which can be related to slower growth that can be advantageous for
455 survival (Sánchez-Gómez et al., 2006). The observed variation among ecotypes seems to be
456 driven largely by the adaptation of a given ecotype to longitude and elevation (temperature
457 and precipitation). Diameter and leaf area increased with longitude (Figure 1, $R^2 = 0.52$, $P <$
458 0.05 , $R^2 = 0.39$, $P < 0.05$, respectively). Low values of leaf area may be considered as an
459 advantageous morphological mechanism for adaptation of the semiarid ecotypes to
460 Mediterranean dry-summer conditions (Valladares et al., 2008; García-Nogales et al., 2016).
461 Smaller and more sclerophyllous leaves are better adapted to higher radiation and
462 temperatures (Costa-Saura et al., 2016). Regarding SLA and leaf thickness, our results show
463 less plasticity in the differences among ecotypes than for leaf area, plant height, or stem
464 diameter, something that was not anticipated as SLA frequently exhibits greater plasticity
465 than other morphological traits (Quero et al., 2008). This low variability of SLA is related to
466 leaf thickness, which - together with leaf area - determines SLA. Hence, when leaves grow
467 under identical natural conditions, variations in leaf thickness tend to be minimised, at the
468 expense of SLA. By contrast, the variability in leaf area was significant. This reflects
469 ecological differences among localities, which result in higher physiological efficiency in the
470 presence of specific stress factors. At the western sites, the favourable environmental
471 conditions led to greater variability in leaf morphology, enabling these ecotypes to attain

472 higher growth rates (Table 3, see stem diameter). The consistency of this geographic cline in
473 *Q. ilex* is strongly suggestive of its global adaptive significance (Sánchez-Gomez et al., 2006;
474 Peguero-Pina et al., 2014). Several authors have described in situ variation in morphological
475 traits with latitude and elevation within *Q. ilex* (Costa-Saura et al., 2016; García Nogales et
476 al., 2016) and greater growth of *Q. ilex* ecotypes from increasing longitudes has been reported
477 (Laureano et al., 2016).

478 The physiological traits measured behaved similarly, showing statistical differences among
479 ecotypes (Table 4). All ecotypes showed low values of water potential (ranging from -2.2 to $-$
480 4.3 MPa), which has been recognised as a common feature of *Q. ilex* populations (Sala and
481 Tenhunen, 1994; Varone et al., 2015). The Ψ_m values reflect the Mediterranean climate in
482 which the ecotypes grow. This was particularly evident for some ecotypes growing in sites
483 with severe water stress (e.g. Almería and Granada with Ψ_m much lower than -2.3 MPa;
484 Table 4, Figure 1, $R^2 = 0.28$, $P < 0.05$), in contrast with the ecotypes from moderate climate
485 conditions (e.g. Pozoblanco and Huelva with Ψ_m below -4.0 MPa, Table 4), which showed
486 the most negative values of water potential. Ecotypes of *Q. ilex* from drier sites typically have
487 a higher water potential, because elevated temperatures induce an increase in photosynthetic
488 and drought tolerance (Varone et al., 2012).

489 Similarly, concerning photosynthesis, the values of photosynthetic assimilation of the eastern
490 ecotypes were higher than those of ecotypes from the western limits, indicating earlier
491 stomatal regulation of the water losses (Brodribb and Hoolbrook, 2003), consistent with the
492 observed lower values of water potential. In contrast, higher values of photosynthesis in the
493 eastern ecotypes imply a great capacity to take advantage of favourable conditions of soil
494 availability and low water potential, to maintain net carbon assimilation for longer. Fleck et
495 al. (1998) reported similar stomatal conductance and photosynthetic rates in *Q. ilex* leaves in

496 summer. In our study, the higher photosynthetic rates in eastern ecotypes were almost entirely
497 due to higher Gs. The conductance (Gs) and transpiration (Tr) decreased with increasing
498 longitude of the ecotype origin (Figure 1, $R^2 = 0.34$, $P < 0.05$, $R^2 = 0.38$, $P < 0.05$). A trend
499 towards increasing rates of net photosynthesis with increasing latitude of origin has been
500 observed in several plants species (Reich et al., 1996). The studies by Martin-St. Paul et al.
501 (2012) suggest that a greater rate of photosynthesis in eastern ecotypes is an adaptation to a
502 short growing season. Also, higher values of net photosynthesis in ecotypes of *Q. ilex* from
503 increasing longitudes were supported by a higher pigment concentration in leaves (Figure 1,
504 $R^2 = 0.75$, $P < 0.05$) with no changes in SLA. Although photosynthetic rates tended to be
505 higher in the ecotypes that are adapted to lower precipitation, these ecotypes grew less,
506 suggesting the down-regulation of photosynthesis in *Q. ilex* subsp. *ilex* during the summer
507 (Ogaya and Peñuelas, 2006).

508 Other plant traits that are useful for the study of the differences among ecotypes are those
509 derived from chlorophyll fluorescence, consistent with the conclusions of Varone et al.
510 (2015). All ecotypes had similar behaviour from a photochemical point of view (Table 4,
511 Figure 1), with no significant differences in instantaneous fluorescence or quantum yield. The
512 values of photochemical traits decrease under drought conditions (Varone et al., 2012), an
513 expression of the down-regulation of the efficiency of light-energy capture (Abadía and Gil-
514 Pelegrín, 2005). The ecotypes with lower instantaneous fluorescence were Almería (A),
515 Granada (G), and PozoAlcón (Po): this may be a consequence of the photochemical
516 adjustment of *Q. ilex*, to avoid damage to the photosynthetic apparatus during the summer
517 (Ogaya and Peñuelas 2006). In contrast, the ecotypes from the western sites (Benamahoma,
518 Ca1, Calañas, H1, and Almadén, S1) had the highest photochemical efficiency, confirming
519 that they are well adapted to high temperatures. It has been proposed (Niinemets et al., 2005;

520 Peguero-Pina et al., 2009) that elastic adjustment may be a common process through which
521 plants adapt to water-deficient environments, to decrease susceptibility to photodamage.
522 However, in our case, the limited impact of summer climatic conditions on the photosynthetic
523 apparatus might be associated with a lack of water stress at the experimental field location
524 (Baldocchi and Xu, 2007). Varone et al. (2015) showed that the photosynthetic efficiency in
525 non-stressed plants was similar among ecotypes that could be clearly differentiated under
526 stress conditions.

527 This absence of photo-oxidative stress in our *Q. ilex* ecotypes was consistent with the
528 maintenance of stable Chl concentrations (Table 4). As has been observed previously, *Q. ilex*
529 maintains high Chl concentrations throughout the year (Gratani et al., 1998), although there is
530 a decrease from July to September (Faria et al., 1998). The photosynthetic pigment
531 concentrations varied among ecotypes, with plants from eastern populations (Granada, G,
532 Valdepeñas, P, Segura, S) having lower levels of pigments (Chl_b and α and β -carotenes) and
533 higher Chl_R (Chl_a/Chl_b ratios), suggesting a reduction in the size of the light-harvesting
534 antenna. Similar photosynthetic pigment concentrations have been previously reported in *Q.*
535 *ilex* (Faria et al., 1998; Morales et al., 2002).

536 *Ecotype clustering*

537 The results of the morphological and physiological analyses apparently confirm a
538 differentiation among the ecotype groups of *Q. ilex* in southern Spain. In fact, cluster analysis
539 of the *Q. ilex* ecotypes revealed a clear morphological and physiological differentiation as a
540 response to the geographical and ecological gradient of variation in the origins of the ecotypes
541 (Figure 1).

542 This differentiation was confirmed when multivariate analysis was applied using
543 morphological and physiological traits (Table 6, Table S1, Figure 2, and Figure 3). A very

544 high degree of mutual dissimilarity characterised the three ecotype groups that were derived
545 from cluster analysis, based upon morphological and physiological characters. The values of
546 all dimensional/counted, transformed, and observed characters exhibited continuous, albeit
547 weak, trend of variation. Previous work (Valero et al., 2011) using morphological, chemical,
548 and proteomic variation in acorns, showed the presence of significant groups of populations.
549 Cluster analysis clearly delineated three groups of ecotypes. The first group (clusters 2 and 3)
550 includes families from the area of Sierra Morena, growing in acid soils (Jiménez Sancho et
551 al., 1996). These ecotypes were characterised by higher values of morphological traits (height,
552 diameter, and leaf area) and higher pigment concentrations (chlorophyll b and carotenoids)
553 than the remaining ecotypes. The efficiency of biomass accumulation, resulting in greater
554 growth, could be influenced by their natural distribution in a more favourable ecological
555 niche. In this study, a positive response to warm and wet site conditions was evident, as was
556 the case of ecotype S1, which showed higher values of Ψ_m than the other ecotypes of this
557 group, but with lower variability than the eastern ecotypes.

558 The second group is composed of the ecotypes from the most eastern sites, which are
559 dominated by semi-arid climates (less than 500 mm of rainfall, concentrated in short periods,
560 and higher temperatures). The eastern ecotypes were classified together with individuals
561 growing on calcareous substrates and limestone on steep mountains (Jimenez-Sancho et al.,
562 1996). The members of this group exhibited contrasting results for their morphological traits,
563 but they displayed a marked trend in their physiology, with higher photosynthetic rates, low to
564 intermediate pigment contents, and low values of Ψ_m . These ecotypes show strong control in
565 the face of summer drought, but also demonstrated the strongest positive response to the
566 better conditions of the common garden. This might give them a competitive advantage under
567 warmer conditions. This argument is strengthened by the PLS values, providing further

568 evidence of the influence of the key climatic factors (temperature in the dry season,
569 evapotranspiration, and water regime, Figure 4A) on the growth of eastern ecotypes of *Q. ilex*.
570 The third group corresponds to cluster 5, Pozo Alcón (Po). This ecotype is located in the Betic
571 mountains (Sub-betic System). The fact that this ecotype did not form a clearly
572 distinguishable group, given that it was included in clusters with ecotypes from eastern and
573 western sites, not only confirms the high variability of *Q. ilex*, but also indicates that not all
574 ecotypes with the same physiological response necessarily have the same appearance. The
575 ecotype of group 3 was characterised by low values of the photosynthetic traits and more
576 negative water potential, the morphological traits being insufficient to characterize the
577 ecotype.

578 *Multivariable analysis of environmental predictors*

579 The analysis of sPLS resulted in the selection of 47 environmental predictors related to
580 different characteristics that influenced the phenotypic behaviour of the ecotypes. The
581 increases in the temperatures and evapotranspiration rates were correlated with the increments
582 of maximum fluorescence, leaf carotene content, and water potential, and with the decreases
583 in transpiration rate and relative content of chlorophyll a.

584 The local climatic conditions influenced the physiological differences among the saplings, in
585 agreement with the results of Varone et al. (2015). The differences between ecotype grouping
586 in the projected spaces are due to the effect of biophysical variables in the PLS projected
587 matrices. Thus, ecotypes such as Benamahoma or Puerto Serrano showed characteristics in
588 the common garden that do not correspond to the trends shown in the circle of correlations. It
589 is reasonable to assume that Benamahoma shows a genetic pattern similar to that of the
590 cluster 2 ecotypes, given that biophysical conditions of their native stands were similar. Our
591 data were not sufficient to explain the influence of the biophysical variables on genetic

592 expression, but an in-depth analysis of the differences between DA and sPLS demonstrated
593 that the morphological and physiological characteristics of the progeny could be influenced
594 by the environmental conditions of the parental population to different degrees.

595 *Afforestation*

596 The importance of plant provenance in ecological restoration and biological conservation has
597 been shown by several studies (Jimenez-Sancho et al., 1996; Doherty et al., 2017).
598 Traditionally, seeds are collected from local sources to produce seedlings to be planted in the
599 same population area. However, in our study, eastern ecotypes represent a more suitable seed
600 source for reforestation programs in a context of global change as they are better adapted,
601 given model projections for hotter and drier summers, and exhibit more rapid recovery of
602 their growth rate following extreme weather events. Additionally, afforestation programs need
603 to adopt appropriate adaptation measures to respond to the threats of climate change. Eastern
604 ecotypes would be likely to spread in response to the rising temperatures and declining
605 summer precipitation that have been projected; however, the distribution of western ecotypes
606 seems to be more likely to decrease in response to hotter and drier summers (Box and Choi,
607 2000). This is consistent with previous observations in Spain, where the *Q. ilex* distribution is
608 limited by summer drought and soil nutrient availability (Benavides et al., 2016).

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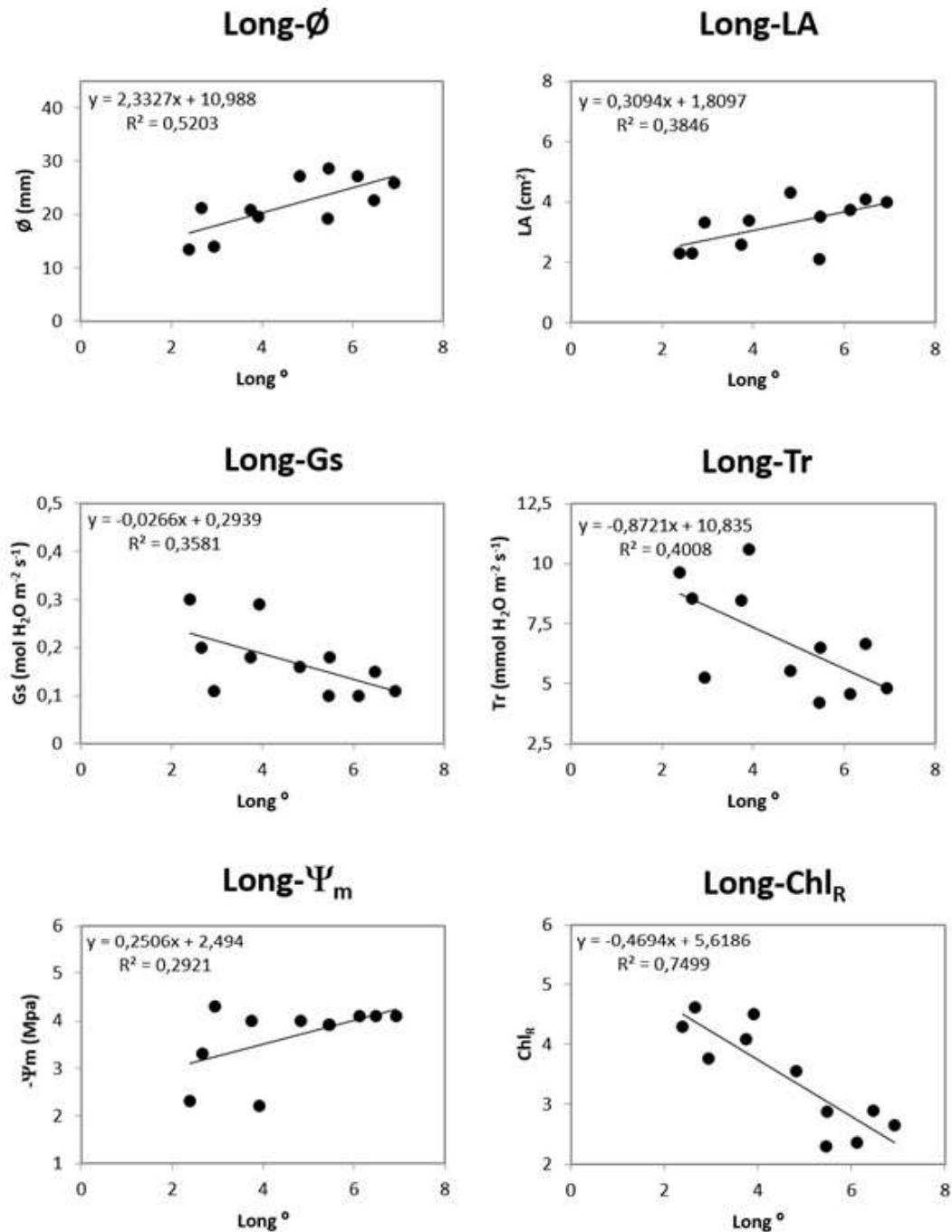
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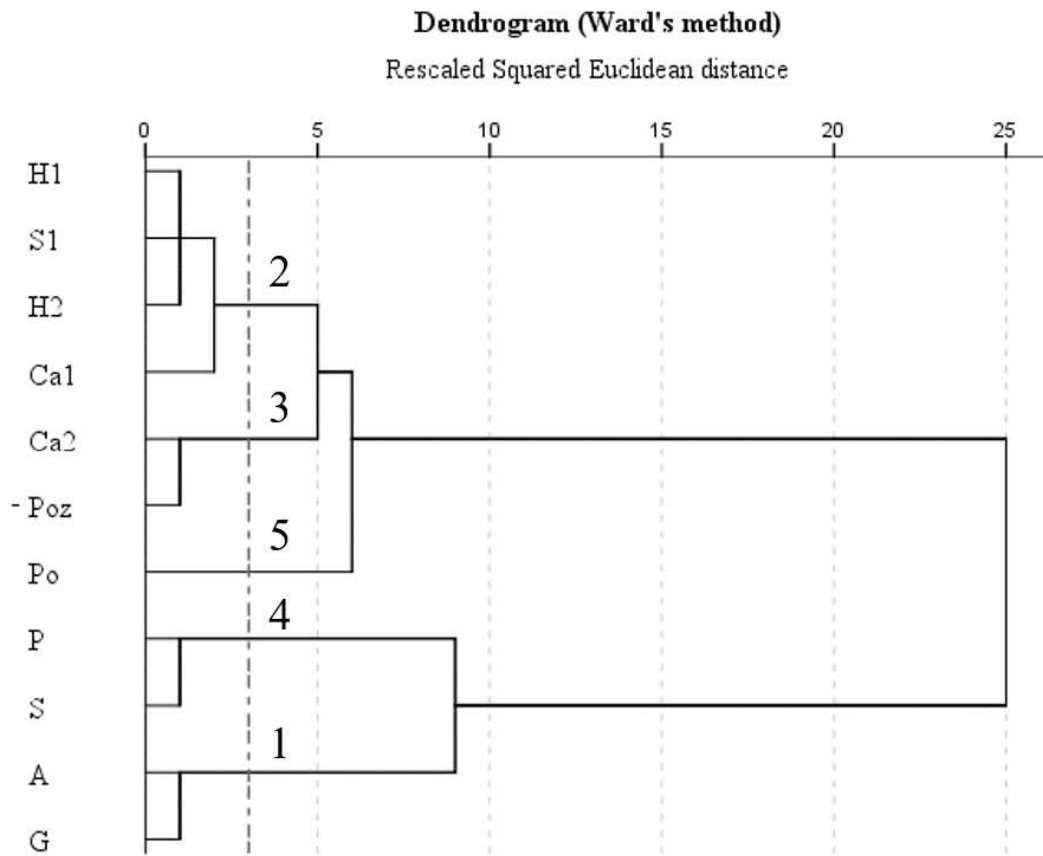


2

3 Figure 1: Linear regressions between longitudinal geographic coordinates (Long) in decimal
4 format (ETRS89) and *Quercus ilex* leaf traits. Ø = collar diameter (mm); LA = leaf area (cm²);
5 photosynthetic assimilation, Gs = stomatal conductance (mmol H₂O m⁻² s⁻¹); Tr = transpiration
6 (mmol H₂O m⁻² s⁻¹); Ψ_m = midday water potential (MPa); CIR = chlorophyll a/b ratio.

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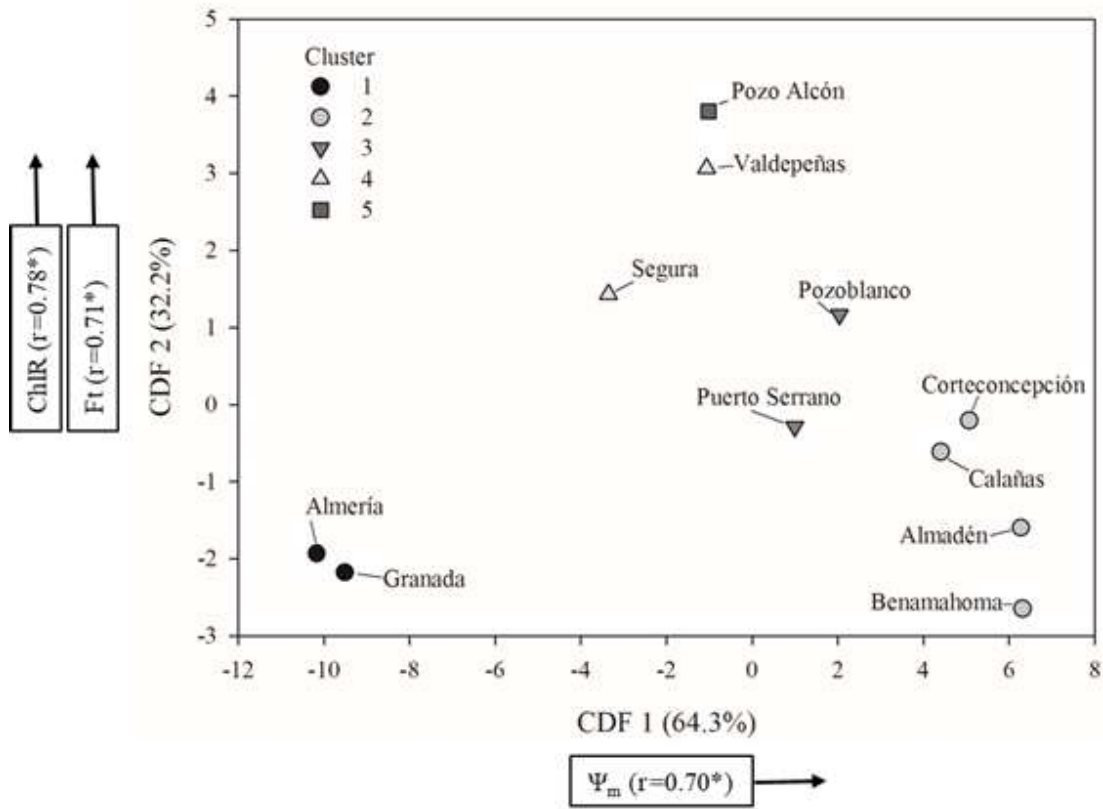


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12 Figure 2: HCA dendrogram of the 11 ecotypes. Numbers identify clusters based upon a
13 natural 5-group division. The bold dashed line shows the approximate rescaled squared
14 Euclidean distance that was selected to delineate the aforementioned clusters. See Table
15 2 for ecotype descriptions.

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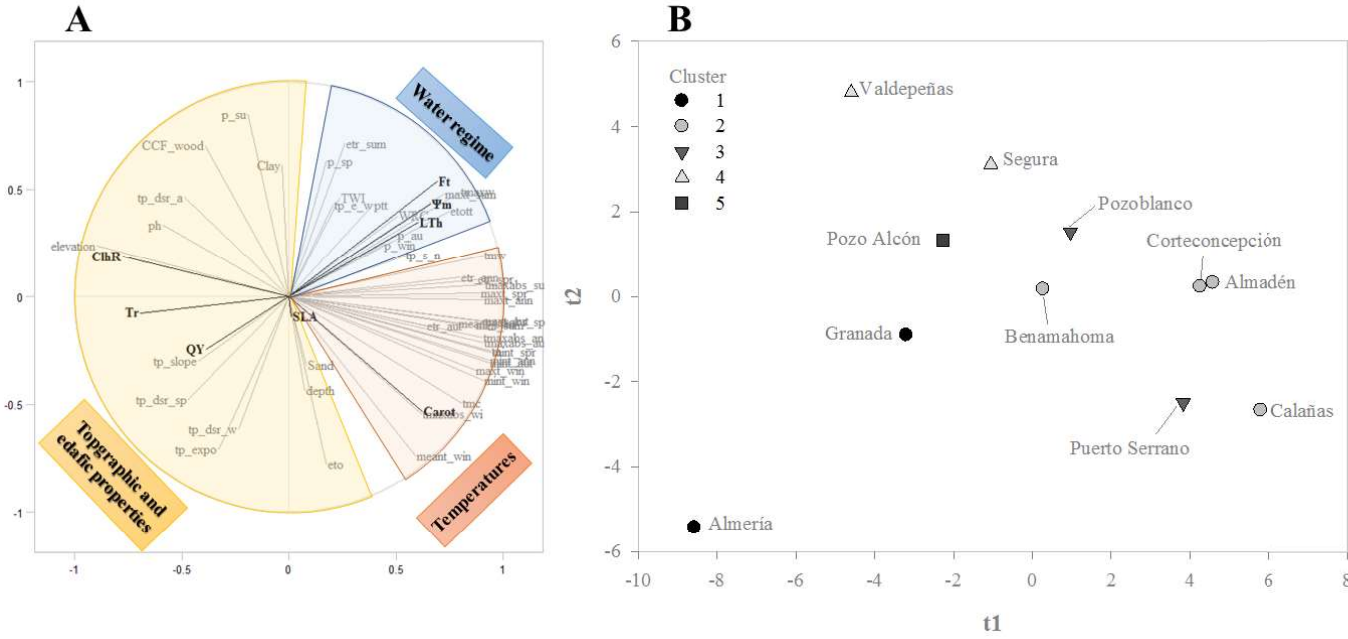


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18 Figure 3: Ordination of the 11 ecotypes in two-dimensional discriminant space; the
 19 position of each ecotype was based on its mean scores that were predicted by the first
 20 and second canonical discriminant functions (CDF). The value that is in parentheses
 21 along each axis is the percentage variance explained by each CDF. The boxes below the
 22 variance explained are variables that are significantly correlated ($P < 0.05$) with each
 23 axis. Values in the legend correspond to clusters that were identified by HCA (Figure
 24 2). See Table 2 for ecotype descriptions.

25

26



27

28 Figure 4: PLS weights plot. A: Pearson's correlation coefficients (r). Black lines
 29 correspond to response variables, light grey lines correspond to predictors. B: Scores of
 30 each case in the projected observation matrix (T). Ecotype classifications correspond to
 31 groups formed by HCA (Figure 2). See Table 2 for ecotype descriptions.

32

- 1 Table 1. Environmental features of *Quercus ilex* ecotypes that were established in a common garden experiment in Hinojosa del Duque
- 2 (Province of Córdoba, southern Spain). Average temperature of the coldest month (Tmin), average temperature of the warmest month (Tmax),
- 3 and average annual rainfall (P) (see ecotype locations in Figure S2, Supplementary Material).

4

Cod.	Ecotype id. / Region. Province	Coordinates (ETRS89)	MASL (m)	T_{max} (°C)	T_{min} (°C)	P (mm)
A	Almería / Sierra de Alhamilla. Almería	36°59'N, 2°05'W	1241	25.2	8.9	277.9
S	Segura / Sierra de Segura. Jaén	38°17'N, 2°36'W	643	23.1	4.4	795.4
Po	Pozo Alcón / Sierra del Pozo. Jaén	30°17'N, 2°36'W	643	24.1	4.4	795.4
P	Valdepeñas / Sierra Sur. Jaén	37°30'N, 3°56'W	618	24.8	5.9	556.3
G	Granada / Arenas del Rey. Granada	36°57'N, 3°54'W	489.3	24.7	11.5	489.3
Poz	Pozoblanco / Valle de los Pedroches. Córdoba	38°22'N, 4°54'W	618	26.8	8.1	612.7
Ca1	Benamahoma / Sierra de Grazalema. Cádiz	36°45'N, 5°27'W	649	24.9	9.8	1263.6
Ca2	Puerto Serrano / Sierra de la Nava. Cádiz	36°54'N, 5°31'W	373	25.5	9.5	1000.5
S1	Almadén / Sierra Norte. Sevilla	37°52'N, 6°05'W	482	26.4	9.5	722.1
H2	Corteconcepción / Sierra de Aracena. Huelva	37°54'N, 6°30'W	369	26.3	5.5	845.6
H1	Calañas / Andévalo Oriental. Huelva	37°52'N, 6°51'W	184	26.5	10.5	635.7

More
western

5

7 Table 2. Variables measured in common garden phenotypic experiment in Hinojosa del Duque (southern Spain).

Code	Variable	Description	Units
TH	Total Height	Height from root collar to upper bud	cm
Ø	Diameter	Diameter of individuals at the root collar	mm
LA	Leaf area	Mean of measured area of 10 leaves per tree	cm ²
LTh	Leaf thickness	Mean of measured leaf blade of 10 leaves per tree	mm
SLA	Specific leaf area	Ratio between LA and leaf dry weight	cm ² g ⁻¹
A	Photosynthesis rate	Mean estimated A of tree leaves	µmol CO ₂ m ⁻² s ⁻¹
Gs	Stomatal conductance	Mean estimated Gs of tree leaves	mmol H ₂ O m ⁻² s ⁻¹
Tr	Transpiration rate	Mean estimated Tr of tree leaves	mmol H ₂ O m ⁻² s ⁻¹
Ψ _m	Midday water potential	Water potential of trees measured at solar midday	MPa
F _t	Instantaneous Fluorescence	Maximum light-adapted fluorescence of chlorophyll	Dimensionless
QY	Quantum yield	Photosystem II efficiency (F _v / F _m - 1)	Dimensionless
Chl _a	Chlorophyll a	Mean estimated Chl a concentration of tree leaves	µg cm ⁻²
Chl _b	Chlorophyll b	Mean estimated Chl b concentration of tree leaves	µg cm ⁻²
Carot	α and β carotenes	Mean estimated carotenes concentration of tree leaves	µg cm ⁻²
Chl _R	Chlorophylls ratio	Ratio between Chl b and Chl a (Chl a Chl b ⁻¹)	Dimensionless

10 Table 3. Morphological variables of ecotypes of *Quercus ilex* that were established in the common garden phenotypic experiment in Hinojosa

11 del Duque (southern Spain). Ecotypes are ordered according to Table 1.

Ecotype	Height (cm)	Diameter (mm)	Leaf Area (cm ²)	Leaf Thickness (mm)	SLA (cm ² g ⁻¹)
A	87.8 ± 10.3 ^b	13.4 ± 1.0 ^b	2.30 ± 0.33 ^{ab}	0.99 ± 0.30	35.51 ± 1.81
S	129.4 ± 13.7 ^{ab}	21.2 ± 2.1 ^{ab}	2.29 ± 0.45 ^{ab}	1.23 ± 0.12	32.59 ± 2.52
Po	89.0 ± 9.7 ^{ab}	14.0 ± 2.6 ^b	3.33 ± 0.53 ^{ab}	1.16 ± 0.23	32.89 ± 3.39
P	125.0 ± 14.8 ^{ab}	20.9 ± 1.5 ^{ab}	2.68 ± 0.29 ^{ab}	1.33 ± 0.17	36.53 ± 2.24
G	101.4 ± 13.1 ^{ab}	19.5 ± 2.4 ^{ab}	3.47 ± 0.51 ^{ab}	1.69 ± 0.17	32.91 ± 0.92
Poz	149.0 ± 13.7 ^a	27.2 ± 1.6 ^a	4.31 ± 0.51 ^a	1.69 ± 0.19	35.72 ± 0.96
Ca1	102.6 ± 9.2 ^{ab}	19.3 ± 0.8 ^{ab}	2.16 ± 0.27 ^b	1.60 ± 0.13	33.27 ± 1.13
Ca2	134.0 ± 4.4 ^{ab}	28.6 ± 0.9 ^a	3.51 ± 0.28 ^{ab}	1.42 ± 0.27	34.67 ± 0.47
S1	125.8 ± 9.7 ^{ab}	27.1 ± 1.7 ^a	3.74 ± 0.42 ^{ab}	1.41 ± 0.24	35.70 ± 0.90
H2	121.5 ± 20.1 ^{ab}	22.6 ± 3.4 ^{ab}	4.10 ± 0.40 ^{ab}	1.63 ± 0.06	33.34 ± 0.32
H1	127.5 ± 8.5 ^{ab}	25.9 ± 3.1 ^a	4.07 ± 0.64 ^{ab}	1.16 ± 0.10	35.52 ± 1.37

12

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Table 4. Physiological variables of ecotypes of *Quercus ilex* that were established in the common garden phenotypic experiment in Hinojosa del Duque (southern Spain).

Ecotype	Stomatal		Water Pot. (MPa)	Inst. Fluorescence (a.u.)	Quantum Yield (a.u.)	Chlorophyll a ($\mu\text{g cm}^{-2}$)	Chlorophyll b ($\mu\text{g cm}^{-2}$)	α & β -carotenes ($\mu\text{g cm}^{-2}$)	Chlorophyll ratio
	Photosynthesis ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	Conductance ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$)							
<i>A</i>	19.35 ± 5.07 ^a	0.30 ± 0.05 ^a	-2.3 ± 0.02 ^c	1.252.9 ± 205.7	0.58 ± 0.04	17.84 ± 5.36	5.05 ± 2.23 ^{ab}	7.04 ± 1.35 ^{ab}	4.29 ± 0.66 ^{abc}
<i>Ca1</i>	8.31 ± 1.43 ^b	0.10 ± 0.02 ^c	-3.9 ± 0.12 ^{abc}	1.860.7 ± 49.5	0.54 ± 0.01	16.35 ± 2.96	7.34 ± 0.34 ^a	8.99 ± 0.60 ^a	2.30 ± 0.46 ^d
<i>Ca2</i>	14.17 ± 1.97 ^{ab}	0.18 ± 0.02 ^{abc}	-3.9 ± 0.07 ^{abc}	1.545.7 ± 115.3	0.57 ± 0.02	17.10 ± 3.44	5.84 ± 0.74 ^{ab}	8.35 ± 1.05 ^a	2.88 ± 0.20 ^{bcd}
<i>G</i>	19.64 ± 1.18 ^a	0.29 ± 0.02 ^{ab}	-2.2 ± 0.39 ^{bc}	1.393.4 ± 79.2	0.56 ± 0.03	9.56 ± 2.12	2.22 ± 0.59 ^b	4.09 ± 0.58 ^b	4.50 ± 0.21 ^{ab}
<i>H1</i>	8.67 ± 1.16 ^b	0.11 ± 0.02 ^c	-4.1 ± 0.12 ^a	1.746.8 ± 109.0	0.50 ± 0.03	18.33 ± 2.06	6.97 ± 0.67 ^a	9.18 ± 0.73 ^a	2.65 ± 0.14 ^{cd}
<i>H2</i>	12.93 ± 1.21 ^{ab}	0.15 ± 0.01 ^c	-4.1 ± 0.09 ^a	1.874.2 ± 160.4	0.49 ± 0.01	16.13 ± 0.24	5.83 ± 0.48 ^{ab}	8.40 ± 0.17 ^a	2.89 ± 0.26 ^{bcd}
<i>P</i>	12.21 ± 2.78 ^{ab}	0.18 ± 0.04 ^{abc}	-4.0 ± 0.17 ^{ab}	1.682.7 ± 121.9	0.53 ± 0.06	8.52 ± 2.41	2.24 ± 0.81 ^b	4.02 ± 0.62 ^b	4.08 ± 0.27 ^{abc}
<i>Po</i>	7.23 ± 2.92 ^b	0.11 ± 0.05 ^c	-4.3 ± 0.16 ^a	1.533.9 ± 129.4	0.47 ± 0.04	13.91 ± 2.85	4.19 ± 1.16 ^{ab}	6.46 ± 1.15 ^{ab}	3.76 ± 0.50 ^{abcd}
<i>Poz</i>	11.79 ± 2.55 ^{ab}	0.16 ± 0.03 ^{bc}	-4.0 ± 0.18 ^{ab}	1.813.8 ± 189.4	0.56 ± 0.06	14.30 ± 3.12	4.41 ± 1.10 ^{ab}	6.90 ± 1.25 ^{ab}	3.55 ± 0.31 ^{abcd}
<i>S</i>	15.07 ± 2.16 ^{ab}	0.20 ± 0.03 ^{abc}	-3.3 ± 0.31 ^{abc}	1.770.1 ± 136.7	0.57 ± 0.04	8.41 ± 1.48	1.97 ± 0.40 ^b	3.58 ± 0.57 ^b	4.62 ± 0.63 ^a
<i>SI</i>	8.38 ± 1.33 ^b	0.10 ± 0.02 ^c	-4.1 ± 0.13 ^a	1.834.6 ± 182.3	0.53 ± 0.04	15.19 ± 1.24	6.59 ± 0.62 ^a	8.37 ± 0.50 ^a	2.37 ± 0.18 ^d

Table 5. Correlations between morphological and physiological variables that were measured in *Quercus ilex* saplings. Correlation coefficients below the diagonal are Pearson correlations (r); those above the diagonal are Spearman rank correlations (ρ). Correlations significantly greater than zero are in bold. Variable descriptions are in Table 2.

	TH	\emptyset	LA	LTh	SLA	A	Gs	Tr	Ψ_m	F_t	QY	Chl _a	Chl _b	Carot	Chl _R	Lat	Long	MASL
TH	-0.202
\emptyset	0.730**	-0.051
LA	0.399**	0.274*	0.131
LTh	0.105	0.072	0.149	0.171
SLA	0.043	0.097	0.123	-0.229	0.057
A	0.182	0.073	-0.037	0.105	-0.089	-0.637**
Gs	0.171	0.033	-0.072	0.061	-0.088	0.935**	-0.696**
Tr	0.137	0.046	-0.177	0.005	-0.036	0.839**	0.912**	...	-0.607**
Ψ_m	-0.443**	0.126	0.123	0.287*	0.309*	-0.441**	0.131	0.434*	-0.300*
F_t	0.214	0.225	0.256*	-0.147	0.223	-0.227	-0.301*	-0.258*
QY	0.344**	0.24	0.102	-0.016	0.303*	0.500**	0.477**	0.432**	...	0.223
Chl _a	0.121	0.138	0.184	-0.033	-0.054	0.024	-0.028	-0.198	...	0.025	0.12
Chl _b	0.115	0.18	0.23	0.018	-0.06	-0.154	-0.247	-0.405**	...	0.148	-0.009	0.860**
Carot	0.107	0.209	0.239	0.04	-0.062	-0.178	-0.267*	-0.412**	...	0.109	-0.005	0.905**	0.932**
Chl _R	-0.070	-0.168	-0.257*	-0.055	-0.008	0.392**	0.513**	0.592**	...	-0.321*	0.229	-0.339**	-0.709**	-0.561**
Lat	0.289*	0.157	0.212	0.011	0.022	-0.145	-0.182	-0.086	...	0.272*	-0.090	-0.168	-0.204	-0.186	0.131
Long	0.260*	0.513*	0.383**	0.182	0.086	-0.265*	-0.391**	-0.368**	...	0.312*	-0.127	0.309*	0.527**	0.567**	-0.625**	-0.047
MASL	-0.275*	-0.478**	-0.387**	-0.151	0.009	0.230	-0.351**	0.239	...	-0.250	0.165	-0.077	-0.208	-0.248	0.370	-0.107	-0.761**	...

*= Significant correlation at $P < 0,05$

**= Significant correlation at $P < 0,01$

Table 6: Results of principal component analysis (PCA) of morphological and physiological that were selected for further multivariate analysis. † Variables that exhibited the highest correlations on each component. Correlations significantly greater than zero are in bold.

	Communalities	Component matrix			Loadings		
		PC1	PC2	PC3	PC1	PC2	PC3
<i>TH</i>	0.633	0.026	0.756†	0.246	-0.281	0.806	0.088
<i>Tr</i>	0.789	0.849†	0.197	0.169	0.554	0.425	-0.262
<i>F_t</i>	0.942	-0.381	0.584	-0.676†	0.049	0.212	0.979
<i>QY</i>	0.731	0.391	0.757†	0.063	0.149	0.813	0.182
<i>Carot</i>	0.827	-0.696†	0.22	0.543	-0.992	0.266	-0.268
<i>Chl_R</i>	0.780	0.872†	-0.125	-0.062	0.790	0.041	-0.187
Explained Variance					37.75 %	26.48 %	14.14 %
Cumulative Variance					37.75 %	64.23 %	78.37 %

1

2 Table S1: Canonical discriminant function (CDF) analysis of five groups that were based a
3 priori upon HCA and which include as predictors the variables that are listed in the table.

4 The values within the table are correlations between the individual variables and
5 discriminant scores for a particular CDF axis. *Significant correlations ($r < 0.05$) are shown

6 in boldface type.

7

<u>Canonical Discriminant Function Axis</u>			
	<u>CDF1</u>	<u>CDF2</u>	<u>CDF3</u>
<i>TH</i>	-0.013	0.331	-0.626*
<i>Ψ_m</i>	0.699*	-0.498	-0.513
<i>Ft</i>	0.081	0.717*	-0.208
<i>QY</i>	-0.115	0.262	0.958*
<i>Carot</i>	0.21	-0.309	0.380*
<i>Chl_R</i>	-0.223	0.783*	-0.581
Eigenvalues	29.914	14.976	1.598
Expl. Variance	64.3 %	32.2 %	3.4 %
Cum. Variance	64.3 %	96.6 %	100 %

8

9

10 Table S2: Predictors that were included in PLS (N = 63) regression and their correlations
 11 with the response variables (based upon Pearson's product-moment correlations), after
 12 sparsity analysis and selection of predictor variables (X matrix).

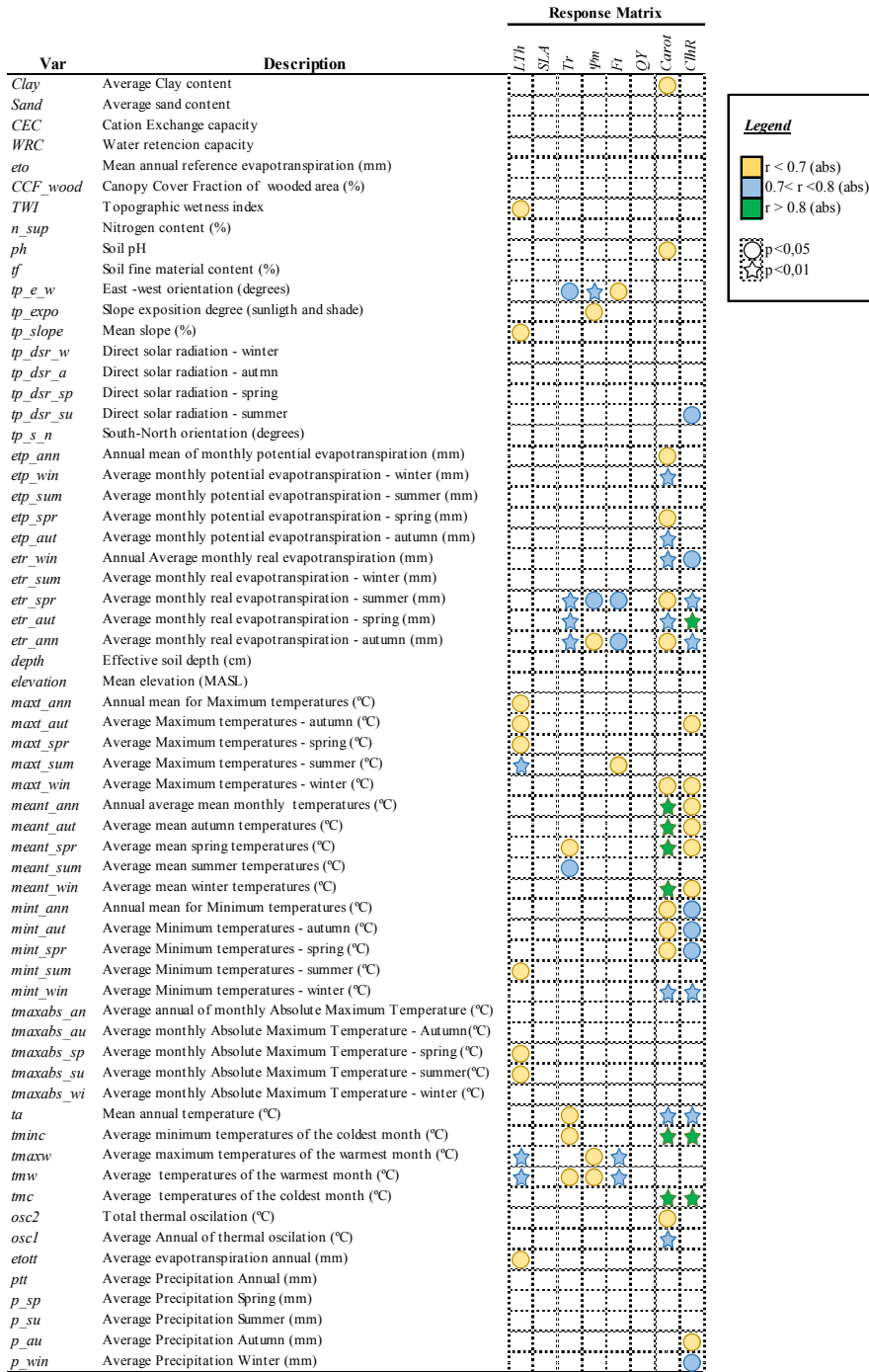
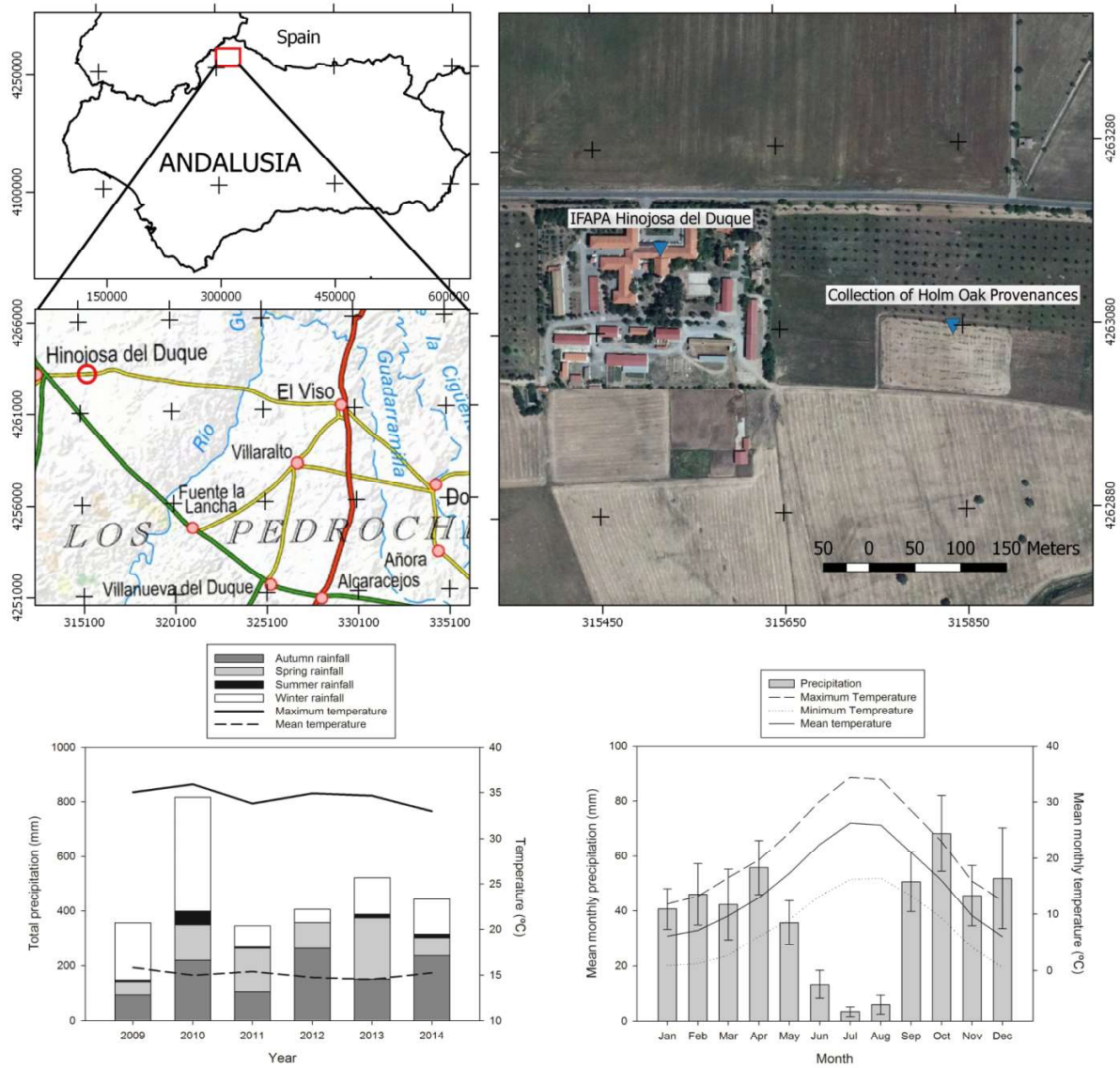


Table S3: Final selection of predictor matrix after sparsity analysis (N=47).

Code	Description
<i>Climatic</i>	
<i>eto</i>	Mean annual reference evapotranspiration (mm)
<i>etr_sum</i>	Average monthly real evapotranspiration - winter (mm)
<i>etr_spr</i>	Average monthly real evapotranspiration - summer (mm)
<i>etr_aut</i>	Average monthly real evapotranspiration - spring (mm)
<i>etr_ann</i>	Average monthly real evapotranspiration - autumn (mm)
<i>elevation</i>	Mean elevation (MASL)
<i>maxt_ann</i>	Annual mean for Maximum temperatures (°C)
<i>maxt_aut</i>	Average Maximum temperatures - autumn (°C)
<i>maxt_spr</i>	Average Maximum temperatures - spring (°C)
<i>maxt_sum</i>	Average Maximum temperatures - summer (°C)
<i>maxt_win</i>	Average Maximum temperatures - winter (°C)
<i>meant_sum</i>	Average mean summer temperatures (°C)
<i>meant_win</i>	Average mean winter temperatures (°C)
<i>mint_ann</i>	Annual mean for Minimum temperatures (°C)
<i>mint_aut</i>	Average Minimum temperatures - autumn (°C)
<i>mint_spr</i>	Average Minimum temperatures - spring (°C)
<i>mint_sum</i>	Average Minimum temperatures - summer (°C)
<i>mint_win</i>	Average Minimum temperatures - winter (°C)
<i>tmaxabs_an</i>	Average annual of monthly Absolute Maximum Temperature (°C)
<i>tmaxabs_au</i>	Average monthly Absolute Maximum Temperature - Autumn(°C)
<i>tmaxabs_sp</i>	Average monthly Absolute Maximum Temperature - spring (°C)
<i>tmaxabs_su</i>	Average monthly Absolute Maximum Temperature - summer(°C)
<i>tmaxabs_wi</i>	Average monthly Absolute Maximum Temperature - winter (°C)
<i>ta</i>	Mean annual temperature (°C)
<i>tmaxw</i>	Average maximum temperatures of the warmest month (°C)
<i>tmw</i>	Average temperatures of the warmest month (°C)
<i>tmc</i>	Average temperatures of the coldest month (°C)
<i>etott</i>	Average evapotranspiration annual (mm)
<i>ptt</i>	Average Precipitation Annual (mm)
<i>p_sp</i>	Average Precipitation Spring (mm)
<i>p_su</i>	Average Precipitation Summer (mm)
<i>p_au</i>	Average Precipitation Autumn (mm)
<i>p_win</i>	Average Precipitation Winter (mm)
<i>Topographic</i>	
<i>CCF_wood</i>	Canopy Cover Fraction of wooded area (%)
<i>TWI</i>	Topographic wetness index
<i>tp_e_w</i>	East -west orientation (degrees)
<i>tp_expo</i>	Slope exposition degree (sunlight and shade)
<i>tp_slope</i>	Mean slope (%)
<i>tp_dsr_w</i>	Direct solar radiation - winter
<i>tp_dsr_a</i>	Direct solar radiation - autumn
<i>tp_dsr_sp</i>	Direct solar radiation - spring
<i>tp_s_n</i>	South-North orientation (degrees)
<i>Edaphic</i>	
<i>Clay</i>	Average Clay content
<i>Sand</i>	Average sand content
<i>depth</i>	Effective soil depth (cm)

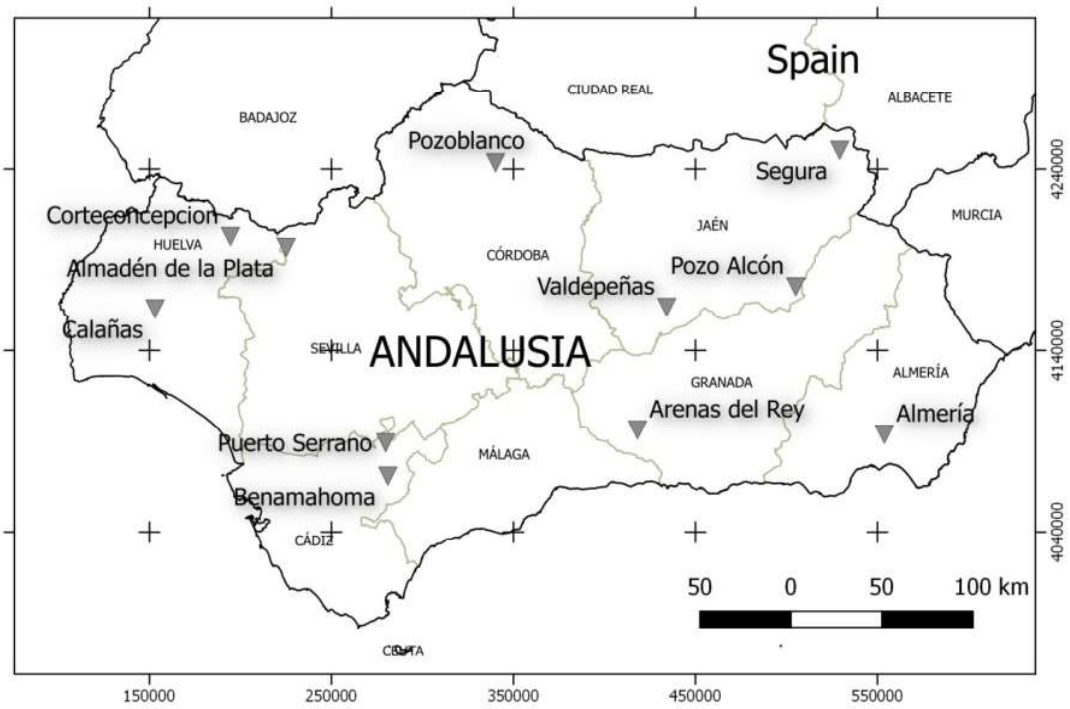
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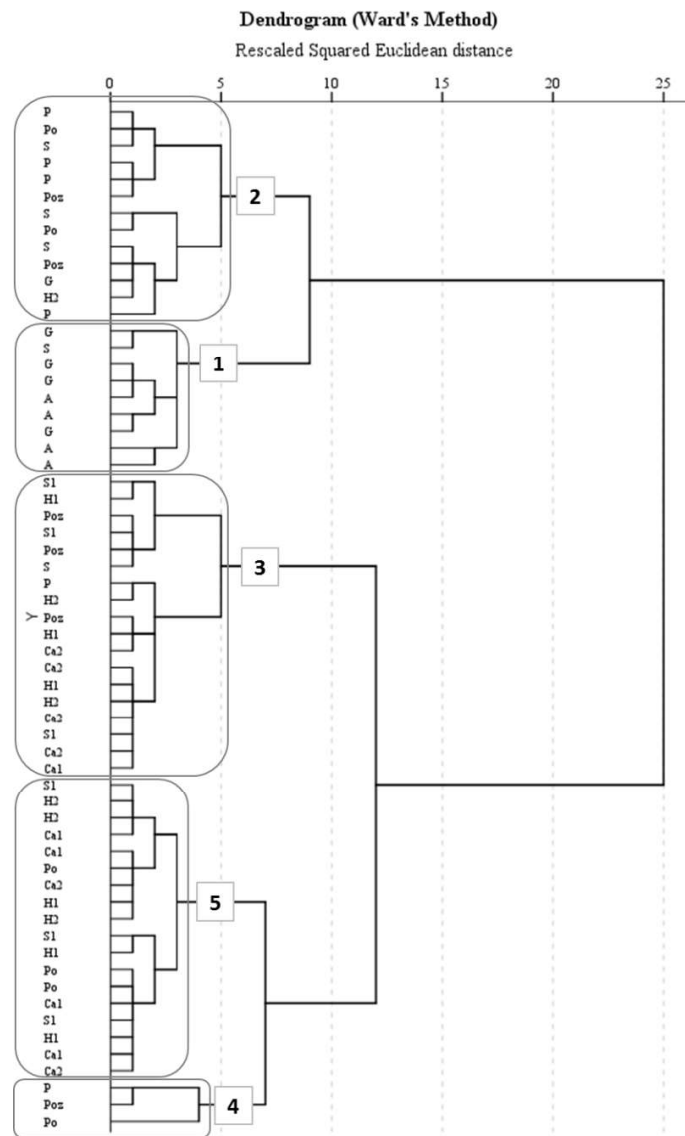
20 Figure S1: Location of the common garden collection in Andalusia, southern Spain (top
21 left). Location of Hinojosa del Duque in relation to other cultural and physical features
22 (middle left). Aerial view of the IFAPA facility (upper right). The two lower panels
23 summarise trends in annual and seasonal meteorological data.



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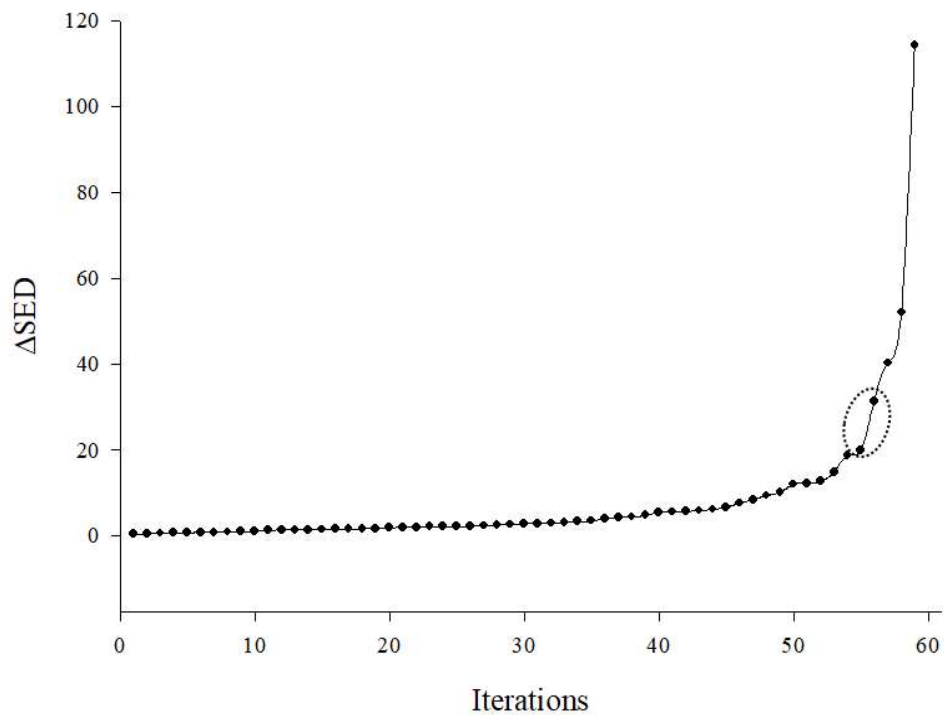
26 Figure S2: Locations of provenance or ecotype populations of holm oak that are included in
27 the Hinojosa del Duque collection.

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30

31 Figure S3: Dendrogram of ecotypes (n = 11, denoted by abbreviations included in Table 1)
 32 and individuals (6 per ecotype) that were measured, compared and agglomerated into
 33 increasing larger groups using Ward's method. Five natural groups (each identified by
 34 number and circled) emerged from the analysis. Dashed lines running perpendicular to the
 35 X-axis show the approximate Rescaled Squared Euclidean Distance between adjacent
 36 individuals, groups of individuals and larger clusters.



37

38 Figure S4: A plot of the difference in sums-of-squares error (Δ SSE) that is associated with
 39 the Squared Euclidean Distance between clusters versus agglomeration step (Iteration). At
 40 step 1, each observation ($n = 66$) is its own cluster and the Squared Euclidean Distance is
 41 the mean value across all individuals. With each increasing iteration, cluster size becomes
 42 larger and cluster number becomes smaller along the X-axis. Natural groupings coincided
 43 with the abrupt change in (Δ SSE), i.e., the maximum derivative of the slope of curve
 44 between iterations 56 and 57 (Circled).

45