# Adaptive diversity and drought tolerance in *Castanea sativa* assessed through genic markers (EST-SSR)

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**Abstract**

Increasing drought conditions in Mediterranean countries are negatively impacting the survival and productivity of *Castanea sativa* Mill. The study aimed to select EST-SSR markers associated with drought stress developed in *Quercus* spp. and evaluate their transferability and polymorphism in *C. sativa*. Eight EST-SSR markers were selected to examine the adaptive potential of four wild populations of *C. sativa* in relation to drought tolerance. To validate markers, offspring of the study trees were water stressed and their drought tolerance was assessed. EST-SSR markers and leaf wilting of seedlings after drought treatment revealed a north-south gradient of *C. sativa* populations. The heritability value obtained for the 'leaf wilting' trait ($h^2$=0.26±0.08) indicated that selection for drought tolerance is possible. The differentiation coefficient of markers showing neutral selection ($F_{ST}$=0.080) was lower than the quantitative genetic differentiation of populations ($Q_{ST}$=0.28), indicating that selection of drought tolerant trees acted spatially in a heterogeneous manner. When assessing the genetic structure of populations, *FIR080* was identified as outlier locus under positive selection. When assessing the phenotypic tolerance to drought of offspring, *GOT004* and *GOT045* were identified as outlier loci under balancing selection and *FIR059* was identified as an outlier locus under positive selection. *FIR059* showed three private alleles for drought-susceptible individuals and two private alleles for drought-tolerant individuals and could therefore be considered as a candidate marker to predict drought tolerance in unstressed *C. sativa* trees. Combined use of functional markers and phenotypic traits is a powerful approach to determine genetic variation at the adaptive level in *C. sativa*. The results illustrate the potential of EST-SSR markers for early selection of drought tolerant plant material.

**Keywords** Climate change, water stress, heritability, genetic differentiation, chestnut

## Introduction

Global climate change is expected to raise global surface temperature (Duveiller *et al*., 2018) and enhance the frequency and severity of drought episodes (Vicente-Serrano *et al*., 2017), decreasing available water and accelerating the decline and mortality of European forest trees (Bréda *et al*., 2006). The impact of climate change on trees is complex, because diverse combinations of alterations in temperature and precipitation in different tree species can produce varied results and responses (Parmesan, 2006). The ability of forests to react and adapt to environmental changes is mostly due to their elevated intrapopulation genetic diversity and phenotypic plasticity (Jactel *et al*., 2017; Woodcock *et al*., 2018). A population genomics approach provides useful information about how selection can shape patterns of functional genetic diversity (González-Martínez *et al*., 2006; Allendorf *et al*., 2010). Local adaptation and significant differentiation for genes involved in adaptive traits (e.g. drought tolerance) are key elements in enabling adaptability of tree populations to future scenarios (Petit and Hampe, 2006).

Simple sequence repeat (SSR) markers derived from expressed sequence tags (EST) have been increasingly used to assess genetic variation and allow more effective conservation of tree genotypes. They are developed from expressed regions of the genome with known or suggested functions (Scott *et al*., 2000; Krutovskii and Neale, 2001; Kalia *et al*., 2011). Although reported to be less polymorphic than genomic SSRs, these markers are superior in functional diversity in relation to adaptive variation and interspecific transferability (Varshney *et al*., 2005; Yatabe *et al*., 2007). EST-SSRs associated with drought adaptation responses have been evaluated in *Quercus* spp. (Lind and Gailing, 2013; Sullivan *et al*., 2013) and several potential markers have highlighted substantial

differences in adaptation among different oak species, coinciding with their differentiated water stress tolerance.

The European chestnut (*Castanea sativa* Mill.) is a widespread tree species present in forests from Portugal to the Caucasus and southern England to Crete. The distribution of *C. sativa* across the Mediterranean basin explains why it is able to adapt to varying environmental conditions and shows significant genetic differentiation between and within populations (Lauteri *et al*., 1998; Martín *et al*., 2010; Mattioni *et al*., 2017). In southern and western Spain, the productivity and survival of this species are being strongly impacted by arid conditions, temperature increase and water deprivation due to global change. This makes it urgent to evaluate functional genetic diversity associated with drought in populations from these areas to identify tolerant genotypes. Differences in adaptive drought-response traits such as growth, morphology and phenology have been reported in *C. sativa* populations from contrasting climate environments (Lauteri *et al*., 2004; Ciordia *et al*., 2012; Míguez-Soto and Fernández-López, 2014).

Natural selection is the central force in evolution and the mechanism by which individuals adapt to their environments. Specific genomic regions that respond to selection (i.e., differentiation after adaptation to local conditions across populations) are expected to appear as outliers from the pattern observed at the neutral genomic level. Detection of outlier loci with unusually high or low levels of variation is therefore extremely useful to separate genome-wide effects caused by demographic processes from adaptive locus-specific effects (Luikart *et al*., 2003).

In the current climate change scenario, evaluating adaptive genetic variability associated with water stress in *C. sativa* and identifying adapted populations of this tree species are the first steps in its preservation. For this, the objectives of the study were to 1) select EST-SSR markers developed in *Quercus* spp. associated with drought stress and evaluate their transferability and polymorphism in *C. sativa*, 2) use the selected EST-SSR markers to explore the adaptive potential of *C. sativa* populations in relation to drought stress tolerance, and 3) determine whether the selected EST-SSR markers are able to discriminate between trees according to their drought tolerance.

## Methods

*Plant material*

The distribution of *C. sativa* in Spain is discontinuous. Forests are more prevalent and dense in the north than in the centre and south (Figure 1). This tree species grows from sea level to 1,800 m and adapts to a wide range of climate conditions with annual rainfall ranging from 500 to 2,500 mm. In October 2016, 30-39 healthy and vigorous *C. sativa* trees from four natural populations (Bergongo, Hervás, Constantina and Montseny), in areas of contrasting climate conditions throughout Spain were sampled (Figure 1 and Supplementary Table S1). Trees were selected at least 70 m apart to minimise the chances of sampling intercrossed individuals. Samples of five to six healthy green leaves per tree were collected from twigs about 2-3 m from soil level.

To assess the drought tolerance of the four populations, a greenhouse experiment was performed with one-year-old seedlings. The greenhouse was located in Maceda, Ourense (42º16'34''N, 7º37'29''W; 598 m a.s.l.; Figure 1). In November 2016, 12 mother trees per population were randomly selected from the sampled populations. About 100 seeds

per tree were collected by hand and stored in a cold chamber at 4ºC for two weeks. Seeds were sunk in water and those that failed to float were discarded as non-viable. The remaining seeds were immersed in a fungicide solution (2 g $L^{-1}$ Thiram 80GD, ADAMA Inc., Spain) for 10 min, rinsed, then stratified in moistened blond peat (Pindstrup Mosebrug Inc., Spain) for two months at 5ºC.

*Genetic analysis*

A total of 749 EST-SSRs designed on EST from *Quercus robur* and *Quercus petraea* and associated with bud phenology and drought stress (Durand *et al*., 2010; Ueno *et al*., 2010) were initially screened. Markers specifically developed from sequences from genes associated with water stress were selected. For this, functional annotations of EST-SSR markers were obtained by searching in the non-redundant NCBI database (e-value of $1e^{-6}$), using as search option 'EST sequences database'. To identify accurately the putative function associated with water stress, the UniProt Knowledgebase (UniProtKB) was used and functional information on proteins (molecular description, taxonomy and sequence data) and literature associated were considered. The analysis showed that 295 EST-SSR markers had no known function, 383 had functions not associated with water stress and only 71 were associated with water stress. Twenty of these 71 EST-SSRs were preselected based on the information provided about their polymorphism, linkage group and potential transferability to *C. sativa* (Durand *et al*., 2010; Bodénès *et al*., 2012, Supplementary Table S2).

Genomic DNA was extracted from 18-20 mg lyophilised leaves according to the Qiagen DNeasy™ Plant mini Kit protocol. To test transferability and polymorphism, DNA from 12 *C. sativa* trees was amplified and the amplification products were run on agarose gel.

Ten of the 20 EST-SSRs amplified and only eight of these showed polymorphism in all samples (Table 1). Based on the size of the products, two multiplex-PCR mixtures were designed, the first including *FIR080*, *GOT004*, *GOT021* and *VIT057* primers and the second including *FIR059*, *FIR094*, *GOT045* and *VIT033* primers (Table 1). The forward primers were labelled with a fluorochrome (6-FAM, VIC, NED, PET; Applied Biosystems, Foster City, California, USA). The amplification was carried out in 20 μL total volume containing 20 ng genomic DNA following the Qiagen multiplex kit protocol. Cycling parameters were 15 min at 95°C, 30 cycles of 30 s at 94°C, 90 s at 57°C and 1 min at 72°C, and a final step of 30 min at 72°C.

Amplification products (1 μL) were added to 20 μL formamide and 0.3 μL LIZ and denaturated at 95°C for 5 min. The samples were run on an ABI Prism 3130 Avant DNA sequencer. The resulting raw data were collected applying GeneMapper software (Life Technologies). The alleles were determined by automated binning and checked by visual inspection.

*Drought tolerance assessment*

In January 2017, germinated seeds were individually measured (length, width and depth), weighed and planted in 50-cell rigid plastic root trainers (300 mL volume; 18 cm high, 5.3 × 5.3 cm upper surface) containing vermiculite and blond peat (1:5, pH 5.5). Earlier research by Cubera *et al*. (2012) showed that this pot size would provide seedlings with unrestricted root growth during drought treatments. The plants were arranged following a split-plot random design replicated in four blocks, with the watering treatments acting as the main factor (two categories: water-stressed and control; whole plots) and the populations as the split factor (four categories: Bergondo, Hervás, Constantina and

Montseny, as shown in Figure 1 and Supplementary Table S1; split plots). In the four blocks, the four populations were represented in each whole plot by five individuals from the 12 open-pollinated families. Individuals were randomly positioned within the blocks. The experiment comprised 1,920 plants corresponding to 4 blocks × 2 watering treatments × 4 populations × 12 families × 5 individuals and therefore included 480 plants per population and 40 plants per family. Additionally, in each root trainer single ramets were planted of clones P011 (commercial hybrid resistant to *Phytophthora cinnamomi*; González *et al.*, 2011) and Cs-12 (susceptible to *P. cinnamomi*). Time to germination was assessed weekly. Chestnuts from about 25% of the families did not germinate well or yielded fewer than three plants per block. Plants were kept in natural daylight under greenhouse shade that reduced solar radiation by 50% and hand watered every four days to field capacity until they were well established.

On June 5 2017, when plants were approx. 17 cm in height, two treatments were applied: watering three times a week to field capacity (control), watering every 15 days to field capacity (water stress). Soil moisture was checked in 10 cells per block and treatment using a TDR 100 soil moisture meter (Spectrum Technologies Inc., Plainfiel, Illinois, USA) and 12-cm-length rods. Gas exchange of plants checked for soil moisture was assessed at midday using a portable differential infrared gas analyser (IRGA) (LCi, ADC Bio Scientific Ltd., UK) connected to a broadleaf chamber. Measurements on June 19 2017 confirmed differences in soil water content and stomatal conductance ($g_s$) between control and water-stressed plants (0.127±0.029 *vs* 0.041±0.013 $cm^3cm^{-3}$ and 234±25 *vs* 103±19 mmol $CO_2$ $m^{-2}s^{-1}$, respectively). Watering treatments lasted one month. The water stress tolerance of each family was assessed (i) by comparing $g_s$ values in control and water-stressed plants at the end of treatments, (ii) by visual estimation of the leaf wilting

190 percentage in water-stressed plants at the end of treatments, using 10% intervals, and
191 considering leaves and portions of leaves wilted if they were dry, brown in colour, and
192 (iii) by determining plant mortality 15 days post treatments. Plant height was measured
193 before treatments and plant resprouting was recorded throughout the vegetative period.
194
195 *Statistical analysis*
196 Intra- and inter-population genetic diversity indices were calculated using GenAlEx 6
197 (Peakall and Smouse, 2005): number of total alleles per locus (A); effective number of
198 alleles (Ne); observed (Ho), expected (He) and unbiased expected heterozygosity (uHe);
199 and number of private alleles in populations (Pa). The inbreeding coefficient $F_{IS}$ (Weir
200 and Cockerham, 1984) was computed using Arlequin 3.11 (Excofier *et al*., 2005) and its
201 deviation from zero tested by 10,000 allele permutations. Differentiation between
202 populations was calculated by $F_{ST}$ (Weir and Cockerham, 1984) and $R_{ST}$ (Slatkin, 1995).
203 Deficits in heterozygotes attributable to the presence of null alleles were tested for each
204 locus using FreeNA software (Chapuis and Estoup, 2007). LOSITAN software (Antao *et*
205 *al*., 2008) was used to detect outlier loci; i.e., markers in which the genetic diversity
206 within populations (heterozygosity) and between populations ($F_{ST}$) do not conform to the
207 prediction of neutral selection. Similar heterozygosity and $F_{ST}$ values for all loci indicates
208 a shared demographic history, loci showing unusually large amounts of $F_{ST}$ may mark
209 regions of the genome that have been subject to directional selection, and loci with
210 unusually small amounts of $F_{ST}$ may mark regions that have been subject to balancing
211 selection. In this respect, directional or positive selection leads to fixation of the favoured
212 allele in a population, and this allele is responsible for progressive adaptation to new
213 environments. Balancing selection promotes polymorphism by selecting for diverse
214 alleles at the same locus, favouring two or more alleles simultaneously. Balancing

selection is therefore an important force for maintaining genetic variation in populations and allows adaptive evolution when the environment changes. Simulation of neutral selection was conducted under the stepwise mutation model with 50,000 iterations at 95% confidence level and a false discount rate (FDR) of 0.1.

The genetic structure of *C. sativa* populations was analysed by applying a model-based Bayesian approach implemented in STRUCTURE v.2.3.4 software (Pritchard *et al*., 2000) using the admixture model on the whole dataset and the correlated allele frequencies (Falush *et al*., 2007; Hubisz *et al*., 2009). The range of possible number of clusters (*K*) tested was 1 to 7 (putative number of populations plus 3) and six independent runs were performed for each *K* value, with a burn-in period of 10,000 steps followed by $10^5$ MCMC replicates. To identify the number of clusters (*K*) that best explained the data, the rate of change on $L(K)$ ($\Delta K$) between successive *K* values was calculated according to Evanno *et al*. (2005) using STRUCTURE HARVESTER (Earl and vonHoldt, 2012). The six runs for each simulation were averaged using CLUMPP software (Jakobsson and Rosenberg, 2007) and represented graphically with DISTRUCT (Rosenberg, 2004).

To quantify differences in drought tolerance in *C. sativa* among and within populations, three models were built including the variables assessed after drought exposure ($g_s$, leaf wilting and plant mortality). Stomatal conductance of water-stressed plants was expressed as a reduction in $g_s$ in comparison with non-water-stressed control plants for each of the 20 pairs of stressed *vs* non-stressed root trainers, and data were expressed in percentages. Leaf wilting of plants was individually assessed by visually estimating the proportion of dry areas of leaves in relation to the total leaf area, and was expressed in percentages. Angular transformation of the $g_s$ and leaf wilting percentages ($x$) was performed to

normalise data [$y$=arcsine($x$/100)$^{1/2}$]. The first and second models were general linear mixed (GLM) models and included 'transformed $g_s$' and 'transformed leaf wilting' as the dependent variable, respectively. The third was a generalised linear (GLZ) mixed model and included mortality (parameterised as 0 or 1 if the seedling survived or not) as the dependent variable. 'Block' was used as a fixed factor; 'population', 'block × population' interaction and 'mother tree' (nested within 'population') were used as random factors; and 'individual seed weight', 'time to germination' and 'plant height' were used as covariates. Families with fewer than 14 germinating seedlings were not included in the analyses. The residuals of the models were checked for normality and means were compared using the Tukey HSD test. Because the second model was highly significant (Table 3), the genetic structure of the plant material was used to estimate narrow-sense heritability across populations ($h^2$) and population genetic differentiation ($Q_{ST}$) for the 'leaf wilting' trait. Calculations were performed following Solla *et al*. (2016), assuming that native *C. sativa* stands in Spain are self-incompatible (McKay, 1942). Genetic ($r_g$) and phenotypic ($r_p$) Pearson correlations among the variables 'seed weight', 'time to germination', 'plant height', 'leaf wilting', 'plant mortality' and 'plant resprouting' were obtained using family-mean and individual values, respectively. A Bonferroni correction was applied and significances were divided by the number of statistics involved.

To study spatial variation in selection to drought among *C. sativa* populations, quantitative genetic divergence between populations ($Q_{ST}$) and neutral genetic differentiation between populations ($F_{ST}$) were compared. The $F_{ST}$ coefficient was calculated by using only markers that were detected under neutral selection according to the LOSITAN software. If $Q_{ST}$ value is higher than $F_{ST}$, directional selection that favours different genotypes in different populations is inferred, which indicates local adaptation

265 or spatially divergent selection (Saether *et al*., 2007; Ramírez-Valiente *et al*., 2018). If
266 $Q_{ST}$ is lower than $F_{ST}$, the same genotypes are favoured equally in the different
267 populations, which indicates stabilizing selection (Saether *et al*., 2007; Ramírez-Valiente
268 *et al*., 2018). Finally, if the $Q_{ST}$ and $F_{ST}$ estimates do not differ, the observed degree of
269 differentiation in quantitative traits could have been reached by other neutral evolutionary
270 processes.
271
272 To determine whether the selected markers associated with drought were able to
273 statistically differentiate the four populations studied, a discriminant function analysis
274 (DFA) was performed. DFA is a supervised projection method in which *a priori*
275 information about sample grouping in the dataset is used to produce measures of within-
276 and between-group variance. This information is then used to define discriminant
277 functions that optimally separate the *a priori* groups (Martín *et al*., 2008). 'Population'
278 was used as the grouping variable and 'alleles of each individual per primer' (n=16) was
279 used as the independent variable list. To determine whether the selected EST-SSR
280 markers were able to discriminate between trees by 'drought tolerance', a second DFA
281 was performed. *A priori* information about drought tolerance of individuals was obtained
282 from their offspring. Individuals were grouped into drought tolerant (n=8) and drought
283 susceptible (n=13) if their progenies wilted 0-50 or 76-100%, respectively. When
284 performing the DFA, individuals wilting from 51 to 75% (n=20) were not considered
285 because including them implied less signification than if discarded. Individuals were also
286 grouped by $g_s$ and mortality, but the discriminant functions were less significant.
287 'Drought tolerance' was used as the grouping variable and 'alleles of each individual per
288 primer' was used as the independent variable list. In both DFA, forward stepwise analysis
289 and casewise missing data deletion were applied. The discriminant functions were

displayed graphically as score scatter plots to observe any groupings among individuals. Models and analyses were performed with STATISTICA v10 (Stat Software Inc., Tulsa, OK, USA).

## Results

*Genetic diversity of the selected EST-SSRs*

Ten of the 20 EST-SSRs preselected from *Quercus* were successfully transferable to *Castanea*. Eight showed high levels of polymorphism and were used to assess genetic diversity (Table 1). In the 136 *C. sativa* trees, 39 different alleles were identified. Alleles per locus ranged from 2 to 14 (Table 1), with a mean of 4.88 alleles. Allele frequencies were distributed unevenly within the loci, with 14 catalogued as rare (frequency below 5%; *FIR080*, *GOT004*, *FIR059*, *FIR094* and *GOT045*) and three private alleles in *GOT021* and *FIR059* (Table 1).

The *C. sativa* population in Bergondo had the highest level of diversity in number of alleles, expected heterozygosity and number of private alleles (Table 2). Bergondo was the only population polymorphic for the eight markers studied; the other three were monomorphic for *GOT004*. The inbreeding coefficient ($F_{IS}$) was positive and significant in Bergondo and Constantina (Table 2). STRUCTURE software showed that the most probable division with the strongest support in terms of log-likelihood values was detected at $K = 2$ (Figure 2). Based on $K = 2$, Bayesian clustering separated *C. sativa* trees into two main groups corresponding to a north-south geographic pattern, with limited admixture among clusters. The percentage of membership, assessed through the admixture proportion ($Q$) of populations in each inferred cluster, permitted grouping of

Bergondo and Montseny in cluster I ($Q$ values of 0.87 and 0.69, respectively) and Hervás and Constantina in cluster II ($Q$ values of 0.92 and 0.80, respectively) (Figure 2).

*Phenotypic tolerance to drought and validation of EST-SSRs*

Water stress treatment resulted in 32% plant mortality and 64% leaf wilting and reduced $g_s$ by 55-65% in relation to controls. Leaf wilting was the variable that best explained variability in *C. sativa* seedlings to water stress. Leaf wilting differed significantly among populations (Figure 3) and families (Figure 4) and covaried positively with time to germination (Table 3). Models based on plant mortality and $g_s$ were not significant at within-population level and were discarded from further analysis. The model based on leaf wilting provided significant estimates of narrow-sense heritability across populations ($h^2$ = 0.26±0.08) and quantitative genetic differentiation among populations ($Q_{ST}$ = 0.282). Families that had some trees with 100% wilted leaves were able to resprout. This occurred more frequently in trees from Bergondo and Hervás than from Constantina and Montseny (30 and 17% *vs* 4 and 3% plant resprouting, respectively; $P < 0.01$) (Figure 4). Resprouting rates were higher in families that wilted more due to water stress than in families that wilted less ($r$ = 0.47; $P$ = 0.002) (Supplementary Table S3). At the family level, seed weight and plant height were predictive of leaf wilting and plant resprouting (Supplementary Table S3). At the individual level, tall plants from heavy early-germinating seeds wilted more than small plants from small late-germinating seeds (Supplementary Table S3).

Clusters of trees from each population were significantly separated by the DFA (Wilks' Lambda test, $P < 0.0001$). Examination of the Function 1-Function 2 score scatter plots showed overlapping between individuals from Constantina and Hervás and between

individuals from Montseny and Bergondo (Figure 5a), in agreement with STRUCTURE software results (Figure 2). The separation between individuals from Constantina and Bergondo was mainly characterised by the Function 1 axis, which showed a negative score gradient for Bergondo and a positive score gradient for Constantina (Figure 5a). Markers *FIR080*, *GOT045*, *FIR094*, *GOT021* and *VIT033* were significantly involved in *C. sativa* population discrimination at $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P = 0.0020$ and $P = 0.0379$, respectively. LOSITAN software identified *FIR080* ($F_{ST} = 0.233$, $P < 0.05$) as outlier marker under positive selection associated with local adaptation to water stress (Supplementary Figure S1a).

Clusters of trees by drought tolerance were significantly separated by the DFA (Wilks' Lambda test, $P < 0.05$). Separation between susceptible and tolerant *C. sativa* individuals (if their progenies wilted 0-50 or 76-100%, respectively) was mainly characterised by the Function 2 axis, which showed a positive score gradient for tolerant individuals and a negative score gradient for susceptible individuals (Figure 5b). Markers *FIR080*, *VIT057*, *FIR059* and *GOT045* were of particular interest because they were found to be significantly involved in differentiating *C. sativa* individuals with different drought tolerances at $P = 0.028$, $P = 0.01$, $P = 0.041$ and $P = 0.023$, respectively. LOSITAN software identified *GOT004* ($F_{ST} = -0.05$, $P < 0.001$) and *GOT045* ($F_{ST} = -0.051$, $P < 0.001$) as outlier loci candidates influenced by balancing selection and *FIR059* ($F_{ST} = 0.106$, $P < 0.05$) as an outlier locus under positive selection (Supplementary Figure S1b). In locus *FIR059*, private alleles were found in the two tree groups defined: 'drought tolerant' and 'drought susceptible' (Supplementary Table S4).

**Discussion**

364 This study reaches three significant results: firstly, through molecular markers and
365 quantitative traits, it quantifies variation in drought response in *C. sativa* across the vast
366 area occupied by this species in Spain; secondly, it validates the use of functional
367 molecular markers in *C. sativa* as tools to assess adaptive genetic diversity to drought;
368 and thirdly, it suggests several loci to be used in marker-assisted selection to identify
369 drought tolerant chestnut trees for the first time.
370
371 *Drought response in C. sativa populations*
372 Bayesian clustering analysis revealed a clear geographic pattern showing two main
373 groups, with northern and southern populations displaying different genetic composition.
374 The significant differences in the level of differentiation among populations from
375 contrasting climate environments in relation to drought could be explained by
376 temperature and precipitation gradient (Pereira-Lorenzo *et al*., 2010). Constantina is the
377 chestnut population with the highest average temperature and Hervás is the population
378 with the lowest mean annual precipitation (Figure 1). Both populations are subjected to
379 severe drought conditions, especially during summer. The population separation observed
380 is in agreement with previous results in which EST-SSRs were used and a north-south
381 population distribution associated with bud burst was reported (Martín *et al*., 2010). DF
382 analysis also detected a geographic pattern in *C. sativa* populations. The clear separation
383 between Constantina and Bergondo populations based on EST-SSR markers (Figure 5a)
384 is in agreement with the significant difference observed in leaf symptoms based on
385 phenotypic measurements. Constantina seedlings had the lowest 'leaf wilting' values
386 after water deprivation and Bergondo seedlings had the highest (Figures 3 and 4),
387 indicating significant variation in drought response in *C. sativa* populations.
388

389 *EST-SSRs to assess adaptive genetic diversity to drought*

390 The eight EST-SSR markers used were successfully transferred to *C. sativa* and showed
391 considerable polymorphism. Although EST-SSR markers are less polymorphic than their
392 genomic counterparts, several studies have indicated the great efficiency of functional
393 markers for assessing adaptive genetic diversity (Luikart *et al*., 2003; Varshney *et al*.,
394 2005). This was demonstrated in *Quercus* ssp. with EST-SSRs associated with adaptation
395 responses to drought (Lind and Gailing, 2013; Sullivan *et al*., 2013) and in *C. sativa* with
396 EST-SSRs associated with bud burst (Martín *et al*., 2010; Cuestas *et al*., 2017; Martín *et*
397 *al*., 2017).

398

399 In Mediterranean type ecosystems, climate change is projected to induce longer summer
400 drought periods and higher frequency and severity of drought events. The differentiation
401 coefficient obtained through markers associated to drought tolerance and classified by
402 LOSITAN as being under neutral selection($F_{ST}$ = 0.080) was lower than the quantitative
403 genetic differentiation of 'leaf wilting' between *C. sativa* populations ($Q_{ST}$ = 0.28),
404 providing evidence that selection acted spatially in a heterogeneous manner (divergent
405 selection *sensu* Ramírez-Valiente *et al*., 2018). Because drought events involve selective
406 pressure, selection of drought tolerant *C. sativa* trees can be expected in contrasting
407 drought scenarios even in short time intervals.

408

409 In relation to the four outlier markers identified, *FIR080* has a putative function as a *Ricin*
410 *B-like lectin EULS3* (Supplementary Table S2). The *Arabidopsis thaliana* EULS3 lectin
411 has recently been linked to the drought stress response and is part of a complex
412 (carbohydrate) signalling pathway related to stomatal movement (Van Hove *et al*., 2015).
413 Locus *GOT004* has a putative function as a *probable aquaporin TIP1-1* (Supplementary

414 Table S2), associated with the transport of water and small neutral solutes across cell
415 membranes in shoots and leaves. Expression of this gene locus was enhanced by water
416 stress, salt stress and exogenous ABA (Liu *et al*., 1994; Sakurai *et al*., 2005). Locus
417 *GOT045* has a putative function as a *probable E3 ubiquitin-protein ligase*
418 (Supplementary Table S2) and was described as playing combinatory roles in controlling
419 drought signalling pathways in *Arabidopsis thaliana* in response to drought stress (Cho
420 *et al*., 2008; Seo *et al*., 2012). Locus *FIR059*, associated with a gene encoding a *DEAD-*
421 *box ATP-dependent RNA helicase* (Supplementary Table S2), was reported to regulate
422 several stress-induced pathways and to have important functions in the cellular response
423 to salinity and dehydration (Vashisht and Tuteja, 2006; Macovei *et al*., 2012).
424
425 Three of the eight EST-SSR markers used here were studied in *Quercus* spp. by other
426 authors, who identified *GOT021* as an outlier locus under divergent selection of *Q. rubra*
427 and *Q. ellipsoidalis* populations (Lind and Gailing, 2013; Sullivan *et al*., 2013). This
428 study identified *GOT021* as a neutral marker, but with private alleles for both tolerant and
429 susceptible *C. sativa* trees (Supplementary Table S4). *GOT004* was identified here as an
430 outlier locus potentially under balancing selection, whereas in *Q. rubra* and *Q.*
431 *ellipsoidalis* it was catalogued as neutral (Lind and Gailing, 2013; Sullivan *et al*., 2013).
432 *VIT057* was identified here and elsewhere (Sullivan *et al*., 2013) as a neutral marker.
433 Previous studies using LOSITAN software included a similar number of microsatellite
434 markers as here (Martín *et al*., 2010; Lind and Gailing, 2013; Sullivan *et al*., 2013;
435 Wojnicka-Poltorak *et al*., 2016). However, a higher number of screened markers is
436 desirable to confirm results and enhance the discriminative power of the software.
437
438 *Selection of drought tolerant chestnut trees*

Drought tolerance is not easy to assess, because it behaves genetically as a polygenic trait and is associated with a complex expression pattern of dehydration-inducible genes (Shinozaki and Yamaguchi-Shinozaki, 1997). Drought tolerance involves mechanisms operating at different spatial and temporal scales (Tardieu *et al*., 2018). Difficulties arise in interpreting drought tolerance rankings by breeders when phenotypic traits commonly used to assess this trait are unrelated, e.g., at the family level leaf wilting did not correlate with plant mortality (Supplementary Table S3). Plant resprouting, usually ignored in most common garden experiments, did not correlate with plant mortality either, and was highest in families that wilted more. All these processes participate in trade-offs between carbon accumulation and the risk of deleterious soil water depletion (Tardieu *et al*., 2018), and *C. sativa* drought tolerance is probably modulated by short- or long-term strategies involving traits with high intraspecific variability. Moreover, there is no information on maternal effects in *C. sativa*, although they are widely known to appear at early ontogenetic stages, affecting early-life traits such as seed mass, time to germination and seedling growth (Solla *et al*., 2011; Vivas *et al*., 2013; Corcobado *et al*., 2017). Maternal effects seem to have a significant impact on how *C. sativa* seedlings tolerate drought, as shown in Table 3 (currently under research by Camisón *et al*.; unpublished results).

Although previous studies on *C. sativa* explored the genetic basis of several adaptive drought response traits (Lauteri *et al*., 2004; Ciordia *et al*., 2012; Míguez-Soto and Fernández-López, 2014), this study estimates for the first time in *C. sativa* a heritability value associated with drought ($h^2$ = 0.26±0.08). Chestnut is one of the most important trees in Europe, and current varieties and rootstocks are highly susceptible to drought stress. In the face of global climate change, selection of cultivars and rootstocks with improved drought tolerance is of paramount importance in rural areas where *C. sativa* is

464 cultivated. The heritability value obtained in this study was lower than the heritabilities
465 of 'collar rot' variables assessed after inoculation with *Phytophthora cinnamomi* (Santos
466 *et al*., 2015), but high enough to indicate genetic control of the variation observed and
467 confirm that selection for tolerance is possible.
468
469 The GLM model based on leaf wilting indicated significant intrapopulation variability to
470 drought in *C. sativa*, with the highest and lowest variation in leaf wilting responses in the
471 Constantina and Bergondo populations, respectively (results not shown). Individuals
472 from Constantina and Hervás will be cloned and used as breeding stock or as sources to
473 produce nuts with moderate levels of drought tolerance. However, given the difficulties
474 in identifying drought tolerant tree phenotypes, these results provide evidence that
475 marker-assisted selection would be easier and less prone to errors caused by
476 environmental variability. DF analysis identified *FIR080*, *VIT057*, *FIR059* and *GOT045*
477 markers as significantly involved in differentiating *C. sativa* individuals with different
478 drought tolerances. *FIR080* showed one private allele for drought susceptible individuals,
479 and *FIR059* showed three private alleles for drought-susceptible individuals and two
480 private alleles for drought-tolerant individuals. Previous evidence and the identification
481 of *FIR059* as the only marker under positive selection indicate that it could be used in
482 marker-assisted selection to predict drought tolerance in unstressed *C. sativa* trees.

**Supplementary material**

Supplementary material is available at Forestry online

**Table S1** Description of the four populations of *Castanea sativa* assessed for adaptive genetic diversity to drought

**Table S2** Characteristics of 20 EST-SSRs preselected and their putative functions

**Table S3** Family-mean (above the diagonal) and individual-seedling (below the diagonal) phenotypic correlations between traits assessed in one-year-old *Castanea sativa* seedlings exposed to severe water stress. Asterisks indicate significance after applying a Bonferroni correction at $P < 0.05/15$ (*) and $P < 0.01/15$ (**) above the diagonal and at $P < 0.05/6$ (*) and $P < 0.01/6$ (**) below the diagonal; ns = non-significant; – = invalid correlation

**Table S4** Private alleles detected in *Castanea sativa* trees grouped into drought tolerant and drought susceptible when progenies wilted 0-50 or 76-100%, respectively

**Figure S1** Comparison of $F_{ST}$ and He in polymorphic loci to identify outliers and potential candidates for selection using LOSITAN software. (a) considering *Castanea sativa* trees grouped into cluster I and cluster II; and (b) into drought tolerant and drought susceptible when progenies wilted 0-50 or 76-100%, respectively. Graphical output shows the simulated confidence area for neutral loci (pale grey shading).

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## Conflict of interest statement

None declared.

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690 **Table 1** Characteristics of the eight EST-SSRs grouped in multiplexes A and B and used to assess adaptive genetic diversity in 136 *C. sativa*
691 individuals from the four populations sampled

| Locus | Primer sequence (5′–3′) | Motif | Dye | Size (bp) | A | Ne | Ho | He | uHe | Pa | $F_{IS}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Multiplex A | | | | | | | | | | | |
| *FIR080* | F: ACCATACCTGGCTTCGATGA | ACC | VIC | 144-185 | 6 | 2.02 | 0.485 | 0.505 | 0.507 | 0 | 0.043 |
| | R: AAGGTGAGTTGGTGGTGGAG | | | | | | | | | | |
| *GOT004* | F: GGGCATATTGATCGCTTAGG | TG | 6-FAM | 245-255 | 3 | 1.05 | 0.051 | 0.050 | 0.051 | 0 | -0.017 |
| | R: TGAGCATTCATACATTCCATGAT | | | | | | | | | | |
| *GOT021* | F: AGAAAGTTCCAGGGAAAGCA | AT | 6-FAM | 97-101 | 4 | 2.33 | 0.691 | 0.571 | 0.573 | 1 | -0.207 |
| | R: CTTCGTCCCCAGTTGAATGT | | | | | | | | | | |
| *VIT057* | F: TCAGCAAAATCCCAACTTTGT | AACTCG | PET | 141-148 | 2 | 1.89 | 0.551 | 0.471 | 0.472 | 0 | -0.168 |
| | R: ACACTTCGCTGTTCCTCGAT | | | | | | | | | | |
| Multiplex B | | | | | | | | | | | |
| *FIR059* | F: GGTGGTTTCCGTGAGCATAG | GA | NED | 141-186 | 14 | 6.59 | 0.699 | 0.848 | 0.851 | 2 | 0.177* |
| | R: TTGCCACACCTTCTCGTTAG | | | | | | | | | | |
| *FIR094* | F: CAAAAGCCTCTCACTCTTGAGC | CT | 6-FAM | 183-197 | 4 | 2.44 | 0.213 | 0.590 | 0.592 | 0 | 0.641* |
| | R: TCAAACCCAAACAAAACGAA | | | | | | | | | | |
| *GOT045* | F: TCAACAAAACCCATTAAACCAA | CT | VIC | 126-146 | 4 | 1.26 | 0.213 | 0.209 | 0.210 | 0 | -0.015 |
| | R: GGATCGGAGTGAAATGGAGA | | | | | | | | | | |
| *VIT033* | F: CATGAAGAACACACACGATGC | CTT | 6-FAM | 79-81 | 2 | 1.73 | 0.176 | 0.421 | 0.423 | 0 | 0.583* |
| | R: TTCGGTGAACTTGAACTAGGC | | | | | | | | | | |

692 A, number of alleles; Ne, effective number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; uHe, unbiased expected
693 heterozygosity; Pa, private alleles; $F_{IS}$, inbreeding coefficient. *significant at the 0.01 level.

694 **Table 2** Genetic diversity parameters for *Castanea sativa* populations obtained from the eight EST-SSR markers

| Population | A | Ne | Ho | He | uHe | Pa | $F_{IS}$ | $F_{ST}$ | $R_{ST}$ |
|---|---|---|---|---|---|---|---|---|---|
| Bergondo, Galicia | 4.00 | 2.38 | 0.429 | 0.471 | 0.477 | 11 | 0.100* | - | - |
| Hervás, Extremadura | 2.88 | 1.95 | 0.314 | 0.328 | 0.333 | 1 | 0.050 | - | - |
| Constantina, Andalusia | 3.00 | 2.27 | 0.379 | 0.437 | 0.444 | 1 | 0.143* | - | - |
| Montseny, Catalonia | 2.63 | 2.03 | 0.408 | 0.441 | 0.448 | 2 | 0.090 | - | - |
| Overall | 3.13 | 2.16 | 0.383 | 0.419 | 0.426 | | 0.096 | 0.093 | 0.068 |

695 A, number of alleles; Ne, effective number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; uHe, unbiased expected
696 heterozygosity; Pa, private alleles; $F_{IS}$, inbreeding coefficient. *significant at the 0.01 level; $F_{ST}$, differentiation among populations according to
697 Weir and Cockerham (1984); $R_{ST}$, differentiation among populations according to Slatkin (1995).

698 **Table 3** Results of the general linear mixed model for analysis of percentage of leaf wilting in *Castanea sativa* seedlings

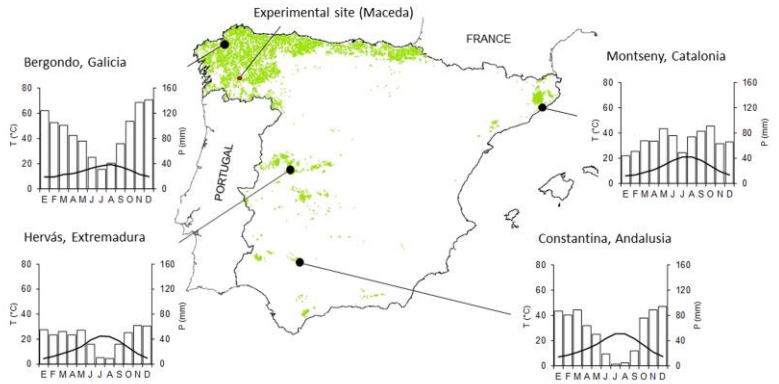
| Effect | Degrees of Freedom | *F*-ratio | *P* value |
|---|---|---|---|
| Random factors | | | |
| Population | 3 | 3.0 | 0.044 |
| Population × Block | 9 | 0.6 | 0.826 |
| Mother tree (population) | 37 | 1.7 | 0.008 |
| Fixed factor | | | |
| Block | 3 | 15.3 | 0.000 |
| Covariates | | | |
| Seed weight | 1 | 0.0 | 0.982 |
| Time to germination | 1 | 12.9 | 0.000 |
| Plant height | 1 | 3.2 | 0.076 |

699 Mother tree (population) = Mother tree nested within population.

700 **Figure Legends**

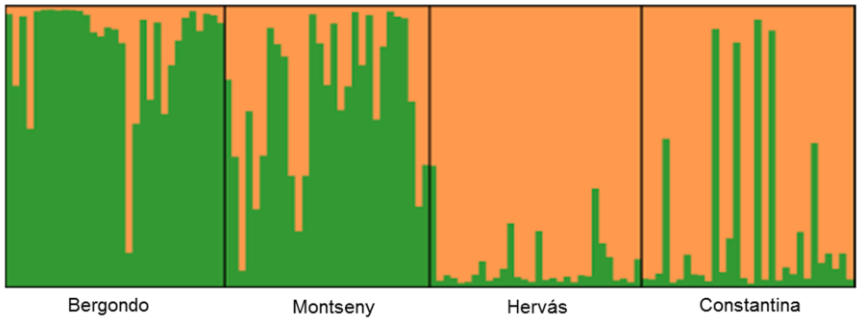
701 **Figure 1** Distribution of *Castanea sativa* in Spain and location (black dots) and

702 climographs of the four study populations.



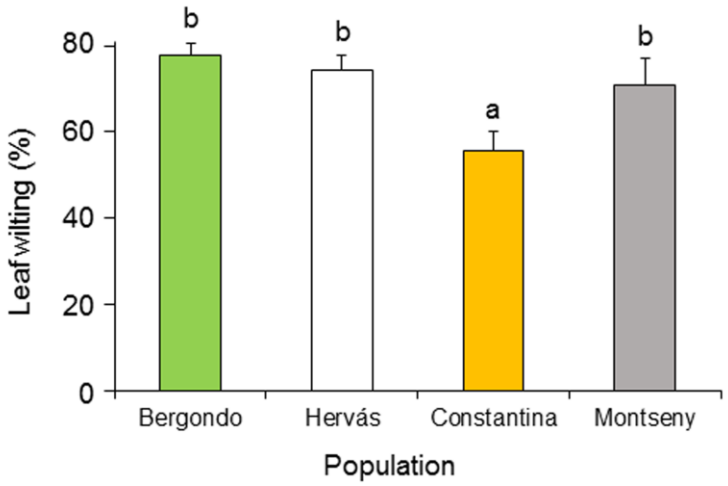

703

704 **Figure 2** Population structure inferred for 120 *Castanea sativa* individuals estimated
705 using STRUCTURE (Pritchard et al. 2000) and data of the eight EST-SSRs for $K = 2$.
706 Each individual is represented by a vertical line and populations are separated by a
707 vertical black line.



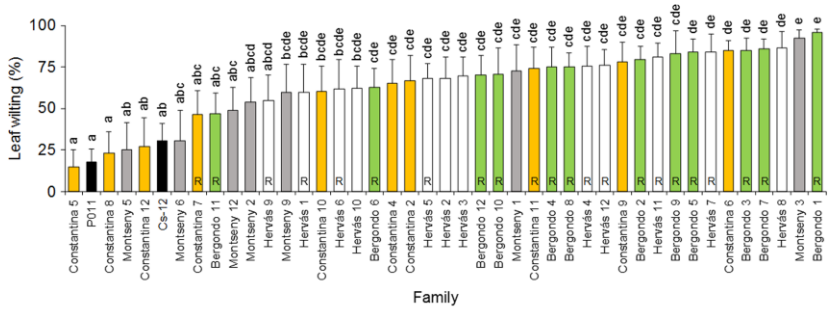

708

709 **Figure 3** Leaf wilting of one-year-old *Castanea sativa* seedlings from different
710 populations after one month of severe water stress treatment. Vertical bars are standard
711 errors and different letters indicate significant differences of mean values between
712 populations ($P < 0.05$) according to the Tukey HSD test.

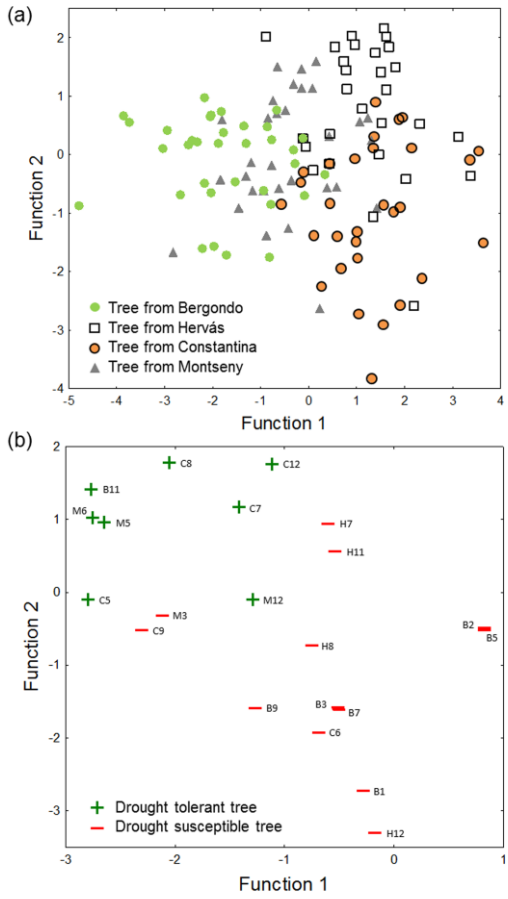



713

**Figure 4** Leaf wilting of one-year-old *Castanea sativa* seedlings from different families and populations after one month of severe water stress treatment. Half of the families included trees with ca 100% wilting that resprouted (R). PO11 and Cs-12 are commercial clones, vertical bars are standard errors and different letters indicate significant differences of mean values between populations ($P < 0.05$) according to the Tukey HSD test.



721 **Figure 5** Discriminant function analysis (DFA) score scatter plots of alleles of eight EST-
722 SSRs of *Castanea sativa* trees from different populations (a) and drought tolerances (b).
723 Drought tolerances of trees were defined based on leaf wilting of progenies after one
724 month of severe water stress treatment (Figure 4). Bergondo (B), Hervás (H), Constantina
725 (C) and Montseny (M).

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