Adaptive evolution of chestnut forests to the impact of ink disease in Spain

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Running title: Evolution of *Castanea sativa* to *Phytophthora cinnamomi*

Abstract *Phytophthora cinnamomi* (*Pc*) is an extremely destructive soil-borne pathogen of Asiatic origin responsible for 'ink disease' in chestnut. This work assesses the adaptive potential to the impact of *Pc* of four Spanish populations of *Castanea sativa* undergoing different selection pressures. To explore the evolvability of *C. sativa* to *Pc* in the selected populations, parameters obtained from neutral and functional genetic diversity were compared with estimates of quantitative genetic variability. Nine EST-SSR markers were selected and their transferability and polymorphism in 137 *C. sativa* individuals were evaluated. To test the potential of EST-SSR markers for early selection of *Pc* tolerant plant material, the offspring of selected individuals were challenged with *Pc*. EST-SSR markers and seedling life expectancy after *Pc* inoculation revealed significant different responses of *C. sativa* populations to *Pc*. The genetic variability observed within populations showed the potential response capacity of Spanish *C. sativa* populations to undergo fast adaptive evolution. The heritability value obtained for the 'life expectancy' variable (h^2 = 0.21±0.11) indicated that selection for resistance to *P. cinnamomi* is possible. Genetic patterns reflected two evolutionarily meaningful groupings of populations, corresponding to the different selective pressure of the oomycete between sites. The differentiation coefficient obtained through markers classified as under neutral selection (F_{ST} = 0.185) was lower than the quantitative genetic differentiation of 'life expectancy' between *C. sativa* populations (Q_{ST} = 0.682), providing evidence that selection acted spatially in a heterogeneous manner. A first link has been identified in trees between population structure and adaptive responses to pathogen-induced selection. The study identified one marker under positive selection that could be used in marker assisted selection to predict resistance to *Pc* in non-inoculated *C. sativa* trees.

Key words: Climate change, biotic stress, *Phytophthora cinnamomi*, heritability, genetic differentiation, chestnut.

Abbreviations: *Phytophthora cinnamomi* (*Pc*).

1 Introduction

Invasive pathogens are drivers of tree evolution. Pathogens encountering susceptible hosts can change the size and genetic structure of tree communities (Agrawal & Stephenson, 1995; Weste et al., 2002), causing a significant drop in gene diversity and triggering multilocus associations in the post-epidemic population (McDonald et al., 1998). Populations with limited phenotypic plasticity and low genetic variation are expected to have difficulty coping with invasive pathogens (Shanjani et al., 2018; Burdon & Laine, 2019). However, little is known about the interplay of evolutionary factors, the impact of invasive pathogens, and the structure of tree populations in shaping the genetic variation and adaptation of susceptible hosts.

Molecular technologies offer new possibilities for population genetic and environmental studies and shed light on the evolution of forests to diseases. Due to their hyper-variability and locus-specific nature, microsatellite markers (simple sequence repeats, SSRs) have become the most commonly used markers for studying population genetic diversity (Tautz & Renz, 1984; Gupta et al., 1996). The location of SSRs in the genome determines their functional role: SSRs found in random genomic regions are selectively neutral, while SSRs found on expressed regions of the genome (expressed sequence tags, EST) with known or putative functions in various physiological conditions of plants are adaptive (Krutovskii & Neale, 2001; Kalia et al., 2011). Information provided by both types of markers is complementary given that neutral SSRs have proven useful for characterization of demographic patterns of variation (migration and drift) and assessment of gene flow, introgressive hybridisation and parental assignment (Allendorf et al., 2010) and adaptive SSRs are directly involved in responses to processes associated to environmental changes and biotic stress. EST-SSRs are described as less polymorphic

than genomic SSRs, but are superior in functional diversity in relation to adaptive variation and interspecific transferability (Varshney et al., 2005; Yatabe et al., 2007). They can be used as a valuable tool to evaluate genetic variation, enabling more effective conservation of tree genotypes, e.g., in the determination of the genetic structure of *Castanea sativa* Mill. based on EST-SSRs associated with bud burst and drought conditions (Martín et al., 2010; Alcaide et al., 2019).

Comparative studies of the divergence of quantitative traits and neutral molecular markers, known as Q_{ST}–F_{ST} comparisons, permit distinction between natural selection and genetic drift as causes of population differentiation in complex polygenic traits (Leinonen et al., 2013). A recent study examining the adaptive potential of four wild *C. sativa* populations in relation to drought tolerance revealed that 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 (Alcaide et al., 2019). Specific genomic regions that respond to natural selection (i.e., differentiation after adaptation to local conditions) are expected to appear as outliers from the pattern observed at the neutral genomic level. Detection of outlier loci (those with unusually high or low levels of variation and differentiation) is therefore useful to separate genome-wide effects caused by demographic processes from adaptive locus-specific effects (Luikart et al., 2003). Several studies have described adaptive evolution of plant pathogens (e.g., Möller & Stukenbrock, 2017; Sánchez-Vallet et al., 2018), but few have addressed changes in the genetic structure of hosts due to pathogens (Shanjani et al., 2018) or evolution of plant-pathogen interactions (Burdon & Laine, 2019).

Phytophthora cinnamomi Rands (*Pc*) is an extremely destructive soil-borne pathogen of Asiatic origin responsible for 'ink disease' in chestnut. In Europe, it is an introduced exotic whose impact was first observed in 1726 ('El Parral', Jarandilla, Extremadura; 40º07'N 5º40'W; Fig. 1) (Elorrieta, 1949), although it was not isolated and identified until 1947 (Urquijo, 1947). In large valleys such as Jerte and Vera, chestnuts have been extensively replaced by *Prunus avium* and remain only on slopes with coarse textured soils, at high altitude. In Spain, the relation between *Pc* presence and disease occurrence reflects a complex interaction among hosts of different susceptibility, and temporal and spatial variability in the environment (Cardillo et al., 2018; Hernández-Lambraño et al., 2018). Occurrence of ink disease is influenced by the high susceptibility of *Quercus ilex* and *Q. suber* to *Pc* (Corcobado et al., 2013; Duque-Lazo et al., 2018), two tree species that dominate forests in southwest Spain. Tree mortality hotspots are widespread in western Spain (Fig. 1) and selective pressures applied by *Pc* resulted in declining chestnut population size and changes in their competitive ability (Camisón et al., 2018). Regeneration of *C. sativa* from seed is possible, but not in southern dry areas, because the root rot caused by the pathogen is exacerbated by the effect of summer drought (Camisón et al., 2018). The Andalusia region of Spain has several disease-free areas, corresponding to spatially discontinuous, patchy sites of chestnut (e.g., Constantina, Fig. 1), whereas Catalonia has very few infested stands. The hybrid *P.* × *cambivora*, associated with ink disease in central and southeast Europe (Vettraino et al., 2005), has not been reported in Spain.

The resistance of *C. sativa* to *Pc* is polygenic and heritable (Santos et al., 2015a; López-Villamor et al., 2018). In the rainy north of Spain, the effect of *Pc* has been mitigated using hybrid rootstocks obtained by crossing local *C. sativa* with the two Asian tolerant

species *C. crenata* and *C. mollissima* (Miranda-Fontaiña et al., 2007; Fernández-López, 2011). In southern regions, use of this resistant rootstock has proven unsuitable because of its low tolerance to cold and drought (López-Villamor et al., 2018). Screening is therefore needed for *Pc* resistance of *C. sativa* populations adapted to continental and Mediterranean conditions.

Molecular characterisation of sequences from genes involved in resistance provides potential for a much deeper analysis of the resistance genetic structure of plant populations than is possible using quantitative genetics. Linkage of phenotypic tolerance to resistance variation at the molecular level is very limited. We aimed to evaluate phenotypic and genetic diversity of four Spanish populations of *C. sativa* undergoing different selection pressures to the impact of *Pc*. The objectives were to 1) confirm the transferability and polymorphism in Spanish *C. sativa* material of EST-SSR markers developed in *Castanea* spp. from expressed genes after inoculation with *Pc*, 2) use the selected EST-SSR markers and inoculated seedlings to compare functional genetic diversity with quantitative genetic variability to *Pc*, 3) explore the evolvability of the Spanish *C. sativa* populations to *Pc*, and 4) test the potential of EST-SSR markers for early selection of *Pc* tolerant plant material.

2 Material and Methods

2.1 Selected populations and plant material

Castanea sativa is widely distributed across Spain, covering approximately 260,000 scattered ha. At the northern margin of its distribution, *C. sativa* development is limited by low temperatures that cause frost damage and reduce seed production, and at the southern margin its survival is limited by drought. Following existing knowledge about

the genetic structure of *C. sativa* (Pereira-Lorenzo et al., 2010; Martín et al., 2012; Alcaide et al., 2019) and information on the distribution of *Pc* in Spain (Corcobado et al., 2013; Duque-Lazo et al., 2018; Hernández-Lambraño et al., 2018), four natural populations of *C. sativa* were selected (Fig. 1; Table S1).

In October 2016, leaves from 30-39 healthy *C. sativa* trees from each population were sampled. Trees were selected with a spacing of at least 70 m to minimise the chances of intercrossing. Samples of five to six healthy green leaves per tree were collected from twigs about 2-3 m from soil level and stored for genetic analysis.

One-year-old seedlings were used to evaluate the response of the four populations to *Pc*. The experiment was performed in a greenhouse in Maceda, Ourense (42º16'N, 7º37'W; 598 m a.s.l.; Fig. 1). In November 2016, 12 mother trees per population were selected at random from the previously genotyped populations. About 100 seeds per tree were hand-picked and stored in a cold chamber at 4ºC for three weeks. Seeds were submerged in water and those that floated 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.

2.2 Genetic analysis of populations

To assess functional genetic diversity within and between the four wild populations of *C. sativa* and examine the adaptive potential of trees in relation to *Pc* resistance , EST-SSR markers associated with chestnut resistance to *Pc* (Santos et al., 2015b) were tested . These markers are sequences that were obtained from differentially expressed genes

(DEGs) identified after inoculation of chestnuts with *Pc* (Santos et al., 2015b; Serrazina et al., 2015) and used to build a genetic linkage map to identify QTLs related to resistance of *Castanea* spp. to *Pc* (Santos et al., 2017b). Based on available information about potential transferability to *C. sativa*, polymorphism, and position on different linkage groups (Santos et al., 2015b, 2017b), seven EST-SSR markers developed in *C. sativa* (*CsPT_0005*, *CsPT_0006*, *CsPT_0008*, *CsPT_0011*, *CsPT_0013*, *CsPT_0020*, *CsPT_0021*) and seven developed in *C. crenata* (*CcPT_0004*, *CcPT_0009*, *CcPT_0014*, *CcPT_0021*, *CcPT_0025*, *CcPT_0028*, *CcPT_0035*) were preselected.

Genomic DNA was extracted from 18-20 mg lyophilised leaves according to the Qiagen DNeasy™ Plant mini Kit protocol. To confirm transferability to *C. sativa* of the seven EST-SSR markers developed in *C. crenata* (described by Santos et al., 2015b) and evaluate polymorphism of the 14 preselected EST-SSRs, DNA from 15 *C. sativa* trees was amplified and the amplification products were run on agarose gel. Ten of the 14 EST-SSRs amplified correctly and only nine of these showed polymorphism in all samples (Table 1). Based on the size of the products, three multiplex-PCR mixtures were designed, the first (A) including *CcPT_0009*, *CsPT_0005*, *CsPT_0006* and *CsPT_0021* primers, the second (B) including *CcPT_0004*, *CcPT_0014* and *CcPT_0035* primers and the third (C) including *CcPT_0021* and *CsPT_0008* primers (Table 1). The forward primers were labelled with a fluorochrome (6-FAM, VIC, NED, PET; Applied Biosystems, Foster City, California, USA). 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 Genescan 500 LIZ and denaturated at 95°C for 5 min. Samples were run on an

ABI Prism 3130 Avant DNA sequencer. The resulting raw data were collected by applying GeneMapper v.4.0 software (Life Technologies). A reference set of alleles were determined and automated binning procedure was applied following the software instructions. The alleles call was checked by visual operator inspection. The related putative genes and functions of EST-SSR markers were identified through BLASTn query against the non-redundant NCBI database (e-value of $1e^{-6}$) (Tables 1, S2). The root transcriptome data sets of *Pc*-inoculated and non-inoculated *C. sativa* and *C. crenata* available in the Short Read Archive at NCBI with the reference PRJNA215368 (Serrazina et al., 2015) were used. To identify accurately the putative function associated with resistance to *Pc*, the UniProt Knowledgebase (UniProtKB) was used and functional information on proteins (molecular description, taxonomy and sequence data) and literature associated were considered.

2.3 Assessment of resistance to *Phytophthora cinnamomi*

The quantitative response to *Pc* of the selected *C. sativa* populations was assessed through artificial inoculations performed in a common garden. Previous experiments with chestnut hybrids conducted under controlled environments reported large additive variation in resistance to *Pc* (Santos et al., 2015a; López-Villamor et al., 2018). In January 2017, germinated seeds were individually 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). Plants were arranged following a randomised block design replicated in four blocks, with 'population' (four categories: Bergondo, Hervás, Constantina and Montseny, as shown in Fig. 1 and Table S1) and 'mother trees' (12 categories) acting as the main factors. Each block included five root trainers. The four populations were represented in each block by five individuals from the 12 open-

pollinated families. Individuals were randomly positioned within the blocks. The experiment comprised 960 plants corresponding to 4 blocks × 4 populations × 12 families × 5 individuals and therefore included 240 plants per population and 20 plants per family. Additionally, in each root trainer single ramets were planted of clones P011 (commercial hybrid resistant to *Pc* and relatively tolerant to drought; González et al., 2011; Alcaide et al., 2019) and Cs-12 (susceptible to *Pc*). Germination was considered successful when the aerial part emerging from the embryo was green. Aerial emergence of plants was assessed weekly. 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 6 July 2017, when plants were approximately 20 cm tall, plant height was measured and roots were inoculated with *Pc*, using a single *Pc* A2 strain (code PS-1683) isolated from roots of a chestnut tree in Bergondo and highly virulent to *C. sativa* seedlings (Camisón et al., 2019). Inoculum was prepared following the procedure described by Jung et al. (1996) and incubated for 5 weeks in Erlenmeyer flasks. Soil was infested using a spoon to carefully mix 12 ml inoculum into the first 3 cm soil of each plant. After inoculation, plants were lightly watered and flooded the following day for 48 h with chlorine-free water to stimulate sporangia production and zoospore release and spread. Every month until the end of the experiment (6 November 2017), plants were waterlogged for 48 h by placing all root trainers individually in large plastic boxes (58 cm × 38 cm × 40 cm, 88 l). Seedling mortality was assessed weekly for 4 months. In November 2017, *Pc* was successfully re-isolated from root samples collected from inoculated plants, following Martín-García et al. (2015).

2.4 Statistical analysis

GenAlEx 6.5 (Peakall & Smouse, 2005) was used to calculate the intra- and inter-population genetic diversity indices: number of total alleles per locus (A), effective number of alleles (Ae), number of private alleles in populations (Pa) and observed (Ho), expected (He) and unbiased expected heterozygosity (UHe). The inbreeding coefficient (F_{IS}) (Weir & Cockerham, 1984) was computed using Arlequin 3.11 (Excoffier et al., 2005) and its deviation from zero was tested by 10,000 allele permutations. Differentiation between populations was calculated by F_{ST} (Weir & Cockerham, 1984) and R_{ST} (Slatkin, 1995). Deficits in heterozygotes attributable to the presence of null alleles were tested for each locus using FreeNa software (Chapuis & Estoup, 2007). LOSITAN software (Antao et al., 2008) was used to identify outlier loci; i.e., markers in which the genetic diversity within populations (heterozygosity) and between populations (F_{ST}) do not conform to the prediction of neutral selection. Similar heterozygosity and F_{ST} values for all loci indicate a shared demographic history, while loci showing unusually large amounts of F_{ST} may mark regions of the genome that have been subjected to positive selection, and loci with unusually small amounts of F_{ST} may mark regions that have been subjected to balancing selection. Neutral selection was simulated under the stepwise mutation model with 50,000 iterations at a confidence level of 95%.

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 5 (putative number of populations plus 1) and 20 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 $L(K)$ (ΔK) between successive K values was estimated according to Evanno et al. (2005) using STRUCTURE HARVESTER (Earl & vonHoldt, 2012). The 20 runs for each simulation were averaged using CLUMPP software (Jakobsson & Rosenberg, 2007) and represented graphically with DISTRUCT (Rosenberg, 2004).

To analyse time-to-death of inoculated plants and compare survival probabilities of *C. sativa* populations to *Pc*, survival time analysis based on the Kaplan–Meier estimate was used (Solla et al., 2011). Survival curves were plotted for the four populations and statistical differences between them were tested by the log rank test. A general linear mixed (GLM) model that included 'life expectancy' of plants as the dependent variable was used to estimate differences in *Pc* resistance in *C. sativa* among and within populations. '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 emerge' 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.

The hierarchical structure of the plant material permitted determination of narrow-sense heritability across populations (h^2) and genetic differentiation among populations (Q_{ST}) for the 'life expectancy' variable. h^2 was estimated as the additive genetic variance (V_A) divided by the phenotypic variance (V_P) (Solla et al. 2016). Assuming that natural *C. sativa* stands in Spain are self-incompatible (McKay, 1942), the additive genetic variance was therefore calculated as 4 times the variance component among families ($\sigma^2_{f\ (pop)}$).

295 Phenotypic variance was deemed to be the sum of $\sigma^2_{f\ (pop)}$, the among-populations
296 variance (σ^2_p) and the error variance (σ^2_e) (Solla et al. 2016). Pooled within-populations
297 narrow-sense heritability was then estimated as:

298 $$h^2 = V_A / V_P = (4 \cdot \sigma^2_{f\,(pop)}) / (\sigma^2_{f\,(pop)} + \sigma^2_{p} + \sigma^2_e)$$

299 Approximate standard errors of heritability estimates were obtained following Jayaraman
300 (1999):

301 $$SE(h^2) \cong \frac{(1-t)(1+nbst)}{\left[(nbs)(f-1)/2\right]^{\frac{1}{2}}}$$

302 where t equals one-fourth of h^2, n is the number of seedlings within a block, b is the
303 number of blocks, s is the number of sites used (if more than one), and f is the number of
304 families. Genetic (r_g) and phenotypic (r_p) Pearson correlations among the variables 'seed
305 weight', 'time to emerge, 'plant height', 'life expectancy' and 'plant mortality' were
306 obtained using family-mean and individual values, respectively. A Bonferroni correction
307 was applied and significances were divided by the number of statistics involved.
308
309 To determine whether the selected EST-SSR markers related to resistance to *Pc* were
310 able to statistically differentiate the four populations studied, a discriminant function
311 analysis (DFA) was performed. DFA is a supervised projection method in which *a priori*
312 information about sample grouping in the dataset is used to produce measures of within-
313 and between-group variance. This information is then applied to define discriminant
314 functions that optimally separate the *a priori* groups (Martín et al., 2008; Alcaide et al.,
315 2019). 'Population' was used as the grouping variable and 'alleles of each individual per
316 marker ' (n = 18) was used as the independent variable list. To determine whether the
317 selected markers were able to discriminate between trees by 'resistance to *Pc* ', a second

DFA was performed. *A priori* information about resistance to *Pc* of individuals was obtained from their offspring. Individuals were grouped into susceptible (n = 11), intermediate tolerant (n = 11) and tolerant (n = 11) if their progenies survived 23 to 34, 35 to 54 or 55 to 100 days post *Pc* inoculation, respectively. These ranges were selected to obtain a similar, balanced number of trees in each group. 'Resistance to *Pc*' was used as the grouping variable and 'alleles of each individual per primer' was used as the independent variable list. Individuals were also grouped by offspring mortality percentages, but the discriminant functions were less significant. In the DFA, forward stepwise analysis and casewise missing data deletion were applied. Models and analyses were performed with STATISTICA v10 (Stat Software Inc., Tulsa, OK, USA).

3 Results

3.1 Population genetic structure based on selected EST-SSR markers

Ten of the 14 EST-SSRs preselected from *Castanea* spp. correctly amplified. Five of the seven EST-SSRs developed from *C. crenata* were successfully transferable to *C. sativa*. Nine markers developed from sequences of genes related to defence responses to biotic stresses showed considerable polymorphism (Tables 1, S2) and were used in the study. FreeNa software showed that the selected markers were not affected by the null allele presence.

In the 137 *C. sativa* trees, 39 different alleles were detected and the number of alleles per locus ranged from 2 to 7 (Table 1), with a mean of 4.33 alleles. Allele frequencies were distributed unevenly within the loci. Nine alleles were classified as rare because their frequencies were below 5% (within *CcPT_0009*, *CsPT_0005*, *CsPT_0006*, *CsPT_0021*,

342 *CcPT_0004*, *CcPT_0014*, *CcPT_0035* and *CcPT_0021*) and three alleles were private
343 within *CcPT_0009* and *CcPT_0004* (Table 1).
344
345 The Bergondo population showed the maximum level of diversity in number of alleles
346 (A = 3.67) and number of private alleles (Pa = 10) (Table 2) and was the only population
347 polymorphic for the nine EST-SSRs evaluated. The other three populations studied were
348 monomorphic for *CsPT_0005*. Only Bergondo had a positive and significant inbreeding
349 coefficient (F_{IS}) with a low value of observed heterozygosity (Table 2). STRUCTURE
350 software identified $K = 2$ as the most probable division with the strongest support in terms
351 of log-likelihood values (Fig. 2). Based on $K = 2$, Bayesian clustering divided *C. sativa*
352 trees into two main groups with limited admixture between clusters (Fig. 2). The
353 percentage of membership, evaluated through the admixture proportion (Q) of
354 populations in each inferred cluster, permitted grouping of Bergondo and Hervás in
355 cluster I (Q values of 0.88 and 0.95, respectively) and of Constantina and Montseny in
356 cluster II (Q values of 0.94 and 0.86, respectively) (Fig. 2).
357
358 **3.2 Phenotypic resistance of *C. sativa* to *Pc* and validation of markers**
359 *Pc* treatment resulted in 73% plant mortality and 58 days total average life expectancy.
360 Survival curves of infected plants differed significantly among populations (Fig. 3A).
361 Life expectancy differed significantly among populations (Fig. 3B) and families (Fig. 3C
362) and covaried positively with seed weight and time to emerge (Table 3). The model based
363 on life expectancy trait provided significant estimates of narrow-sense heritability across
364 populations ($h^2 = 0.21 \pm 0.11$) and quantitative genetic differentiation between populations
365 ($Q_{ST} = 0.68$). Families with higher tree mortality due to *Pc* impact had lower life

366 expectancy rates (Table S3). Also, at the family level, seed weight and plant height were
367 predictive of life expectancy and plant mortality (Table S3).
368
369 Clusters of trees from each population were significantly separated by the DFA (Wilks'
370 Lambda test, $P < 0.0001$). Examination of the Function 1-Function 2 score scatter plots
371 showed overlapping between individuals from Bergondo and Hervás (Fig. 4A), in
372 agreement with STRUCTURE software results (Fig. 2). The separation of Constantina
373 and Montseny individuals those in Bergondo was mainly characterised by the Function 1
374 axis, which showed a positive score gradient for Bergondo and a negative score gradient
375 for Constantina and Montseny populations (Fig. 4A). EST-SSR markers *CcPT_0009*,
376 *CsPT_0005*, *CsPT_0006*, *CsPT_0021*, *CcPT_0004*, *CcPT_0035* and *CcPT_0021* were
377 significantly involved in *C. sativa* population discrimination ($P \leq 0.02$). LOSITAN
378 software detected *CsPT_0005* ($F_{ST} = 0.07$, $P < 0.05$) as outlier locus under positive
379 selection associated with local adaptation to *Pc* (Fig. 5A). Similarly, this locus showed
380 two private alleles (171 and 260), from individuals in the Bergondo population.
381 According to BLASTn query, locus *CsPT_0005* is related with the expression of the
382 pathogenesis-related transcriptional factor *Ethylene-responsive TF ABR1*.
383
384 Clusters of trees by *Pc* resistance were significantly separated by the DFA (Wilks'
385 Lambda test, $P = 0.0018$). Division between susceptible and tolerant *C. sativa* individuals
386 (if their progenies survived 23-34 or 57-100 days post inoculation, respectively) was
387 mainly characterised by the Function 1 axis, which showed a negative score gradient for
388 tolerant individuals and a positive score gradient for susceptible individuals (Fig. 4B).
389 Markers *CcPT_0009*, *CsPT_0005* and *CcPT_0035* were of particular interest because
390 they were found to be significantly involved in differentiating *C. sativa* individuals with

391 different *Pc* tolerances at $P = 0.002$, $P = 0.002$ and $P = 0.001$, respectively. Again,
392 *CsPT_0005* was an outlier locus under positive selection ($F_{ST} = 0.31$, $P < 0.05$) (Fig. 5B),
393 and private alleles were found in this marker for the '*Pc* tolerant' group (Table 4).
394
395 **4 Discussion**
396 Variation in disease resistance is a widespread phenomenon in plant–pathogen
397 associations. The differential response of *C. sativa* provenances to the invasive *Pc*
398 provides a picture of the distinctly different patterns of resistance that could evolve in
399 various areas of the chestnut's natural range. This work highlights significant results: it
400 validates the use of functional microsatellite markers related to *Pc* resistance; it identifies
401 loci under selection as candidates for further marker-assisted selection of tolerant chestnut
402 trees; and it quantifies variation in *Pc* response in *C. sativa* across the vast area occupied
403 by this species in Spain.
404
405 **4.1 Assessment of adaptive genetic diversity to *Pc* and selection of tolerant trees**
406 **using EST-SSRs**
407 Nine EST-SSRs were successfully transferred to our *C. sativa* material and showed
408 considerable polymorphism. Although these markers are less polymorphic than neutral
409 markers, several studies have highlighted their efficiency in assessing adaptive genetic
410 diversity (Varshney et al., 2005; Luikart et al., 2003; Martín et al., 2010, 2017; Cuestas
411 et al., 2017; Alcaide et al., 2019). We identified *CsPT_0005* as an outlier locus potentially
412 under positive selection with private alleles for tolerant *C. sativa* trees. The BLASTn
413 search indicated that *CsPT_0005* is related to a differentially expressed gene with a
414 putative function as an *Ethylene-responsive TF ABR1*, pathogenesis-related
415 transcriptional factor involved in the regulation of plant defence processes. This

transcription factor is a negative regulator of ABA (abscisic acid) signalling pathway in *Arabidopsis thaliana* (Pandey et al., 2005) and its expression allows SA (salicylic acid) and lignin accumulation (Mohr & Cahill, 2007; De Torres-Zabala et al., 2009; Boatwright & Pajerowska-Mukhtar, 2013). Serrazina et al. (2015) reported the relative expression of Ethylene responsive TF ABR1 gene in inoculated and non inoculated Japanese and European *Castanea* genotypes, while Santos et al. (2017a) reported that expression of *Cas ABR1* gene was triggered earlier in the more resistant *Castanea* genotypes after *Pc* inoculation.

DF analysis confirmed *CsPT_0005* marker as significantly involved in differentiating *C. sativa* individuals with different *Pc* tolerances. Moreover, *CsPT_0005* showed two private alleles for *Pc*-tolerant individuals. The above mentioned together with the identification of *CsPT_0005* as the only marker under positive selection suggest it could be used in marker-assisted selection to predict *Pc* resistance in *C. sativa* trees.

4.2 Evolvability of *C. sativa* to the impact of ink disease in Spain

Evolvability is the ability of a biological system to produce phenotypic variation that is both heritable and adaptive. The differentiation coefficient, obtained through the eight markers that were classified by LOSITAN as under neutral selection (F_{ST} = 0.185), was lower than the quantitative genetic differentiation of 'life expectancy' between *C. sativa* populations (Q_{ST} = 0.682). This indicates that selection acted spatially in a heterogeneous manner (divergent selection *sensu* Ramírez-Valiente et al., 2018). Because *Pc* impact involves selective pressure, selection of *Pc* tolerant *C. sativa* trees can be expected in contrasting scenarios with different exposure levels to the pathogen even in short time intervals (Burdon & Laine, 2019).

441

442 Estimated heritability values of traits associated with *Pc* resistance in *C. sativa* × *C.*
443 *crenata* and *C. sativa* × *C. mollissima* progenies (Santos et al., 2015a; López-Villamor et
444 al., 2018) ranged from 0.30 to 0.90. The present study provides a first estimate of
445 heritability of a trait related to *Pc* resistance in wild *C. sativa* populations. Heritability
446 estimates are meaningful parameters in plant breeding. Chestnut is an important tree for
447 nuts in southern Europe, and selection of rootstocks with improved *Pc* resistance is of
448 paramount importance in rural areas where *C. sativa* is cultivated. The heritability value
449 obtained in this study (0.21±0.11) was similar to the heritability value obtained in the
450 same families when assessing tolerance to drought ($h^2 = 0.26 \pm 0.08$; Alcaide et al., 2019).
451 The variance component among families was statistically significant ($P = 0.020$; Table
452 3), indicating genetic control of the variation observed in the 'life expectancy' trait and
453 confirming that selection for resistance is possible (Solla et al., 2014; López-Villamor et
454 al., 2018). Maternal effects (currently under research by our group, e.g., Camisón et al.,
455 2018) appear to have a significant impact on resistance of *C. sativa* seedlings, as shown
456 in Table S3 and reported in other species (Solla et al., 2011; Vivas et al., 2014; Corcobado
457 et al., 2017).

458

459 It is necessary to make a first selection of native *C. sativa* genotypes, not hybrids, that are
460 tolerant to *Pc* and suitable for use in southern and central Spain. Two vigorous *Pc*-tolerant
461 *C. sativa* seedlings from Constantina and one from Hervás are being cloned for further
462 testing for resistance and to aid vegetative propagation and stock–scion compatibility.

463

464 **4.3 Variation of *C. sativa* populations in response to *Pc***

Bayesian clustering analysis revealed two main groups displaying the genetic composition of populations in response to *Pc*. This genetic structure was further partitioned through the DF analysis, which showed a clear separation between the Constantina and Montseny populations (Fig. 4A). The same populations were assessed for drought tolerance using selected EST-SSR markers associated with water stress, and grouped differently: Bergondo and Montseny *vs* Hervás and Constantina ($K = 2$) (Alcaide et al., 2019). Additional Spanish provenances of *C. sativa* were grouped into two gene pools according to neutral markers: Atlantic *vs* southern Mediterranean following a clinal variation ($K = 2$) (Míguez-Soto et al., 2019).

The significant genetic differentiation among populations in response to *Pc* is coincident with the different selective pressure of this oomycete between sites. Galicia and Extremadura are the Spanish regions (Fig. 1) historically most affected by ink disease, in contrast with Andalusia and Catalonia, where occurrence of *Pc* in chestnut is not prevalent. In Catalonian forests, *Pc* has been isolated only twice (Luque et al., 2001; Abad-Campos et al., unpublished results).

The among-population variation in the level of resistance observed could be influenced by the origin of the challenging pathogen isolate. Plant populations are on average 16% more resistant to allopatric pathogen strains than they are to strains occurring within the same population (Laine et al., 2011). However, the strain used was isolated from a chestnut tree from Bergondo, indicating the high capacity of this population to tolerate *Pc*. Selection pressure by the pathogen, the spatial structure of the stand (Rimbaud et al., 2018) or introgression of resistance genes from Asian germplasm already detected for other natural chestnut stands in Galicia (Fernández-Cruz & Fernández-López, 2016;

Perkins et al., 2019) are probably behind the resistance of this chestnut population.

In chestnut stands, *Pc* occurs more frequently in regions with annual rainfall above 1000 mm (Vettraino et al., 2005). Highest resistance to *Pc* was found in Bergondo, in the most humid region of Spain, possibly indicating coevolution between the host and the pathogen. The high variability of response to *Pc* in chestnut could also be influenced by the higher chestnut and pathogen (= disease presence) population sizes. Population size influences the rate of acquisition of a new variation and although mutation rates are independent of population size, large populations will inevitably generate more mutants than small populations. Similarly, larger population sizes enhance sexual recombination. Whether the resistance structure of *C. sativa* populations is determined by pathogen-imposed selection or by other adaptive processes remains unresolved, but we report spatial variation of disease resistance and confirm this variation through genic markers for the first time in chestnut. Tolerance of *Q. ilex* to *P. gonapodyides* has been reported to be geographically dependent, with a trend of increasing tolerance as the environment shifts from arid to humid (Corcobado et al., 2017).

5 Conclusions

This work provides a first link in chestnut between population structure and adaptive responses to pathogen-induced selection. The genetic variability observed within populations indicated the potential response capacity of Iberian *C. sativa* populations to undergo fast adaptive evolution. The markers tested were not under balancing selection, suggesting that selection does not act to maintain different alleles in the gene pool of *C. sativa* populations at frequencies larger than expected under neutral selection. Conversely, *CsPT_0005* locus identified under positive selection could be responsible for

adaptation to *Pc* and could be used in marker assisted selection to predict *Pc* resistance in non-inoculated *C. sativa* trees.

517 **Acknowledgments**

518 The authors are very grateful to M.ª Jesús Fariñas (Tragsa, Maceda), Rubén González,
519 Pilar Santamargarita and Francisco de Dios for technical help, and Jane McGrath for
520 English editing of the manuscript. This study was supported by grant AGL2014-53822-
521 C2-1-R from the Spanish Ministry of Economy and Competitiveness, and the European
522 Union's European Regional Development Fund (ERDF).

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720 **Table 1** Characteristics of the nine EST-SSRs grouped in multiplexes A, B and C and used to assess adaptive genetic diversity in 137 *Castanea*
721 *sativa* individuals from the four populations sampled

Locus	Primer sequence (5′–3′)	Motif	Dye	Size (bp)	A	Ae	Pa	Ho	He	UHe	F_{IS}	Putative function
Multiplex A												
CcPT_0009	F: TTCCACCCAATTGTTACCAC R: GATGATGAAGAAGGGGACGA	(TC)	NED	292-306	6	3.36	1	0.53	0.70	0.71	0.26*	*WRKY transcription factor 22*
CsPT_0005	F: GCTTTTGGTTGATTTGCGAC R: TAAGCCCTGAGAACATTGGC	(AG)	VIC	260-267	3	1.10	0	0.05	0.09	0.09	0.44*	*Ethylene responsive TF ABR1*
CsPT_0006	F: CCTTGCTTCGCTCAGTCATT R: GATCCGACCCGTTTGAGTTA	(TC)	PET	350-365	6	4.21	0	0.58	0.76	0.77	0.24*	*Myb-related protein 44*
CsPT_0021	F: TCTCTTGCATCACCGTCAAG R: GATCCGACCCGTTTGAGTTA	(GGT)	6-FAM	155-168	4	2.86	0	0.50	0.65	0.65	0.23*	*Myb-related protein 44*
Multiplex B												
CcPT_0004	F: GCTGCTTCACAACCTTCCTC R: GCAAGAGATTCCCTTTGCTG	$(CT)_{10}$	6-FAM	346-363	7	3.69	2	0.56	0.73	0.73	0.23*	*Lipoxygenase 3, chloroplastic*
CcPT_0014	F: AGGCGCATTCAAAGAAAGAA R: AGCTGATCAACTCTCGCCAT	(TC)	PET	151-159	3	1.88	0	0.46	0.47	0.47	0.02	*Peroxisomal acyl-coA oxidase 1*
CcPT_0035	F: TTTCTTTGCTTCTTTTGGGC R: ACGCTCCATTACAGCTGCTT	(TTTC)	VIC	201-209	4	1.76	0	0.37	0.43	0.43	0.14*	*Plant cysteine oxidase 2-like*
Multiplex C												
CcPT_0021	F: GCATGCCCATACCCATTAAC R: GGATGCAAAGGCTTTAGCTG	(CCG)	NED	287-298	4	1.49	0	0.25	0.33	0.33	0.25*	*Heavy metal-associated isoprenylated plant protein 3*
CsPT_0008	F: ATGCCCGCAAGATTGTTTAC R: GAACTTAGGTGGCTCAAGCG	(ATG)	VIC	329-332	2	1.79	0	0.44	0.44	0.44	0.01	*SNW domain-containing protein 1*

722 A, mean number of alleles per locus; Ae, mean effective number of alleles per locus; Pa, private alleles by population; Ho, observed heterozygosity;
723 He, expected heterozygosity; UHe, unbiased expected heterozygosity; F_{IS}, inbreeding coefficient; * indicates significant at $P < 0.05$.

724 **Table 2** Genetic diversity using nine EST-SSR markers in the four *Castanea sativa* populations evaluated

Population	A	Ae	Pa	Ho	He	UHe	F_{IS}	F_{ST}	R_{ST}
Bergondo	3.67	1.95	10	0.35	0.41	0.42	0.19*	-	-
Hervás	2.56	1.75	0	0.35	0.35	0.35	-0.04	-	-
Constantina	3.11	2.36	0	0.49	0.52	0.53	0.03	-	-
Montseny	2.89	2.16	0	0.49	0.48	0.48	-0.02	-	-
Overall	3.06	2.05	-	0.42	0.44	0.45	0.04	0.184*	0.230*

725 A, mean number of alleles per locus; Ae, mean effective number of alleles per locus; Pa, private alleles by population; Ho, observed heterozygosity;
726 He, expected heterozygosity; UHe, unbiased expected heterozygosity; F_{IS}, inbreeding coefficient; F_{ST}, differentiation among populations according
727 to Weir and Cockerham (1984); R_{ST}, differentiation among populations according to Slatkin (1995); * indicates significant at $P < 0.01$.

728 **Table 3** Results of the general linear mixed model (GLM) for analysis of life expectancy in *Castanea sativa* seedlings from 4 populations after
729 inoculation with *Phytophthora cinnamomi*

Effect	Degree of freedom	*F*-ratio / χ^2	*P*-value
Random factors			
Population	3	12.2	< 0.001
Population × Block	9	0.6	0.799
Mother tree (Population)[†]	29	1.7	0.020
Fixed factor			
Block	3	0.4	0.723
Covariates			
Seed weight	1	5.6	0.019
Time to emerge	1	11.3	< 0.001
Plant height	1	0.0	0.900

730 [†]Mother tree nested within population.

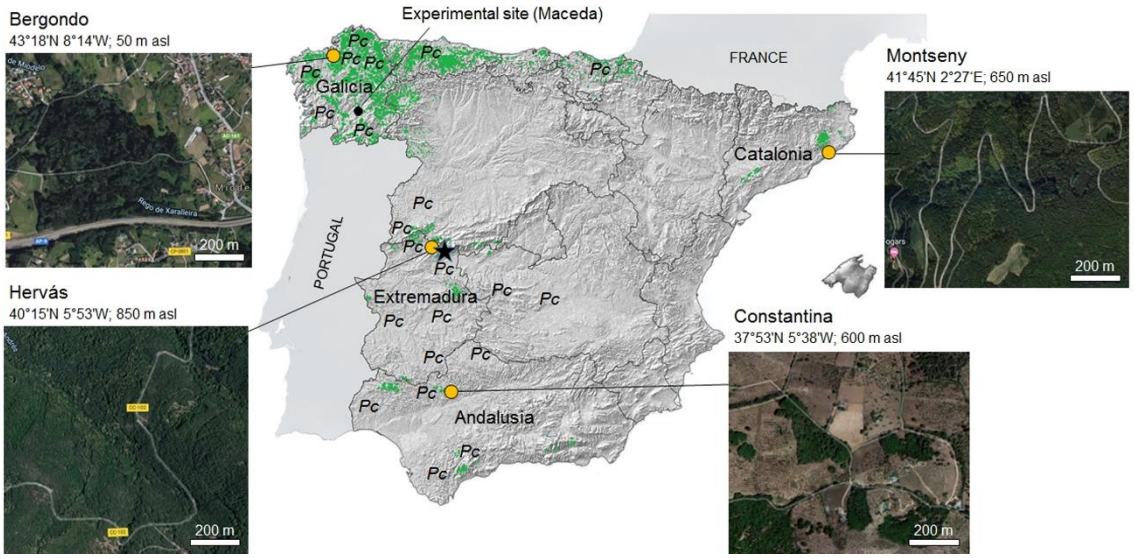
731 **Table 4** Private alleles detected in *Castanea sativa* mother trees grouped into *Phytophthora cinnamomi* resistant and *P. cinnamomi* susceptible if
732 their progenies survived 23-34 or 57-100 days post inoculation, respectively

	Private alleles (bp)	
EST-SSR loci	'*P. cinnamomi* resistant' tree group	'*P. cinnamomi* susceptible' tree group
CcPT_0009	298	302, 304
CsPT_0005	171, 260	-
CsPT_0021	165	155
CcPT_0004	350, 352, 363	346
CcPT_0014	157	-
CcPT_0035	-	201
CcPT_0021	287	-

733

Figure legends

Fig. 1. Distribution of *Castanea sativa* in Spain (green areas) and location of the four study populations, the experimental site and the location where *Phytophthora cinnamomi* damage was first reported in Europe (star). Hotspots of *P. cinnamomi* (*Pc*) are shown according to the literature (Vettraino et al., 2005; Corcobado et al., 2013; Duque-Lazo et al., 2018; Hernández-Lambraño et al., 2018) and unpublished reports.



742 **Fig. 2.** Population structure inferred for 137 *Castanea sativa* individuals estimated using
743 STRUCTURE (Pritchard et al., 2000) and data of the nine EST-SSRs for $K = 2$. Each
744 individual is represented by a vertical line and populations are separated by a vertical
745 black line.
746

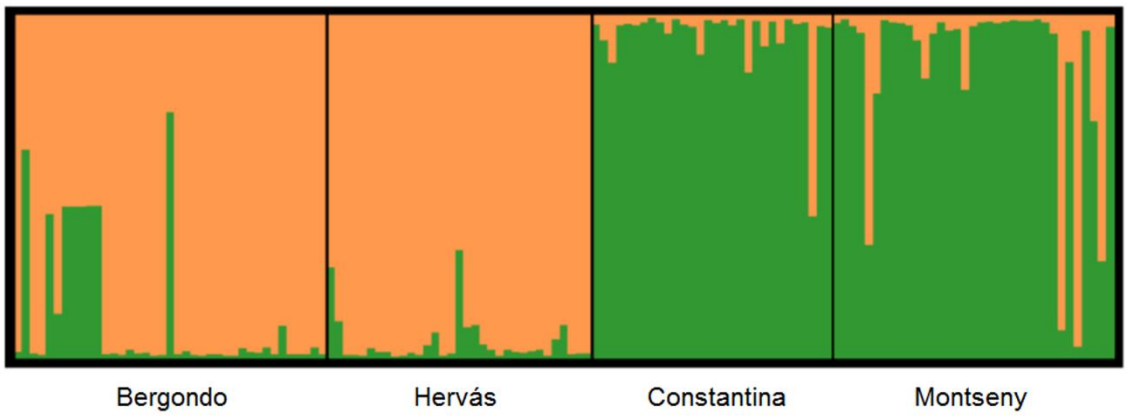


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Fig. 3. Results from one-year-old *Castanea sativa* seedlings inoculated with *Phytophthora cinnamomi*. **(A)**, survival probabilities of seedlings from four populations ($P < 0.001$). **(B)**, mean life expectancy of seedlings from four populations. **(C)**, mean life expectancy of seedlings from different families. P011 and Cs-12 are commercial clones. Horizontal bars are standard errors and different letters indicate significant differences of mean values between populations and families ($P < 0.001$ and $P < 0.05$, respectively) according to the Tukey HSD test.

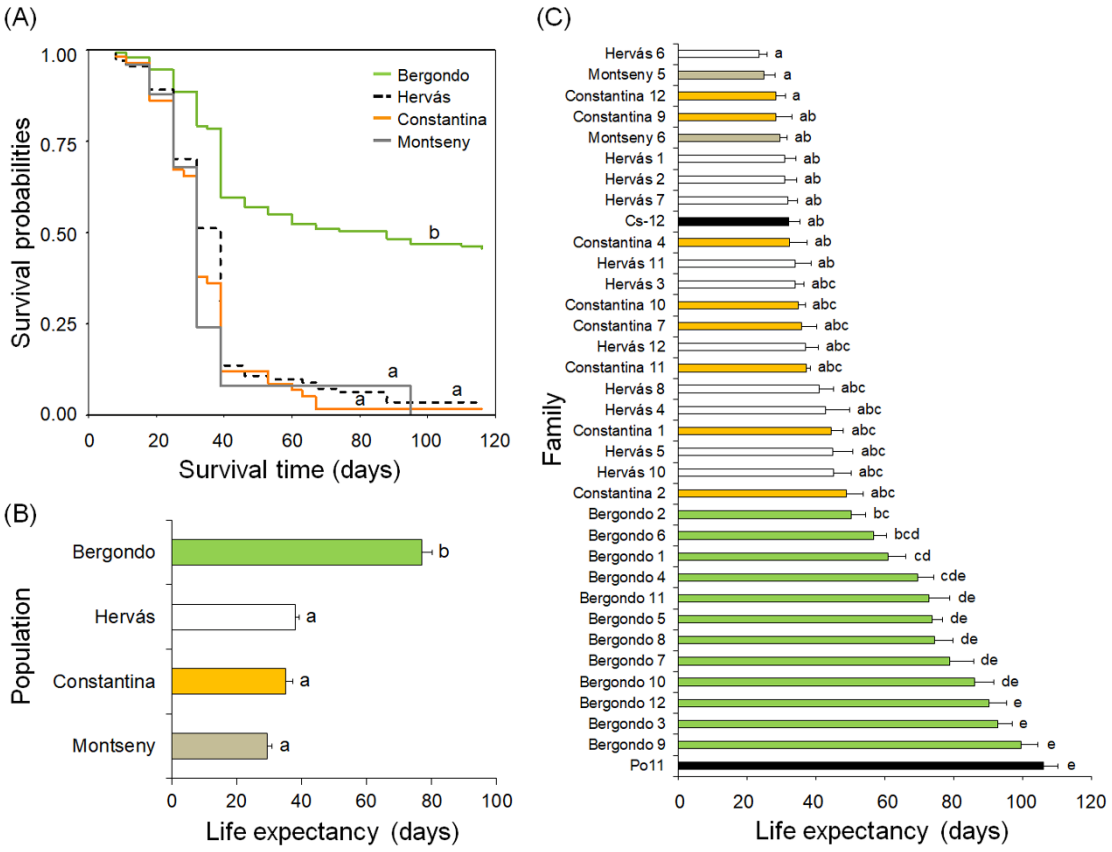

Fig. 4. Discriminant function analysis (DFA) score scatter plots of alleles of nine EST-SSRs of *Castanea sativa* trees from different populations **(A)** and *Phytophthora cinnamomi* resistance **(B)**. *P. cinnamomi* resistance of trees were defined by progeny life expectancy after inoculation (Fig. 3C). Bergondo (B), Hervás (H), Constantina (C) and Montseny (M).

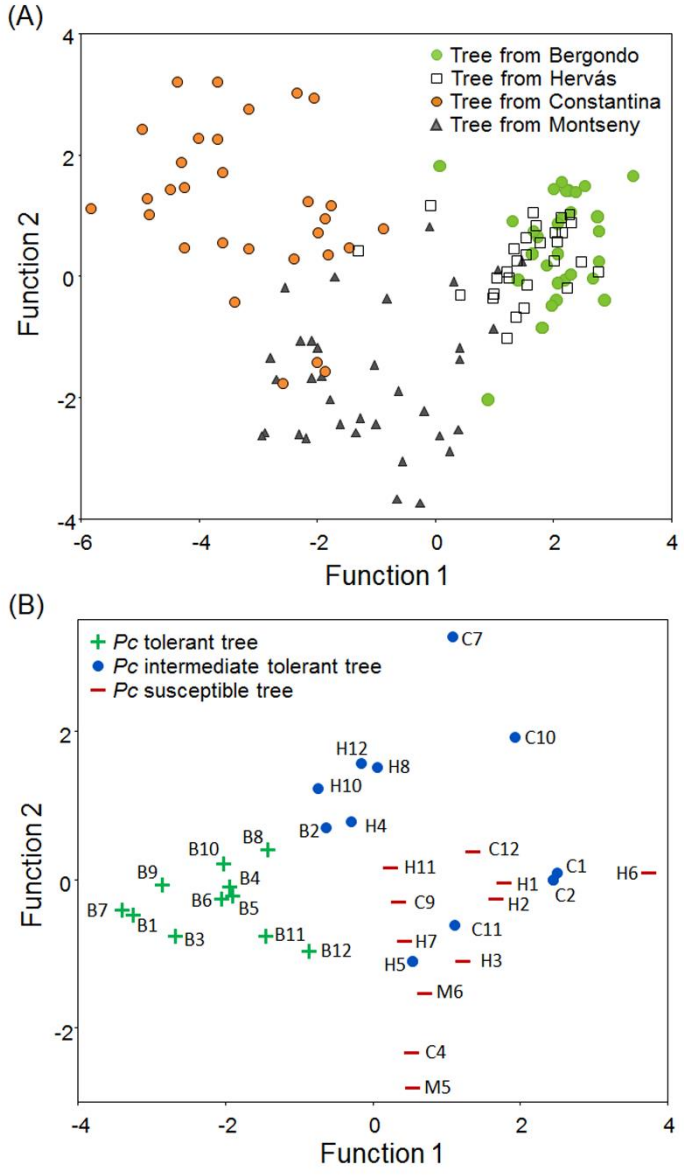

Fig. 5. F_{ST} and He comparisons in polymorphic loci to identify outliers and potential candidates for selection considering *Castanea sativa* trees grouped into cluster I and cluster II **(A)**, and considering *C. sativa* trees clustered into *Phytophthora cinnamomi* tolerant and *P. cinnamomi* susceptible when progenies survived 23-34 or 57-100 days post inoculation **(B)**, respectively. Graphical output shows the simulated confidence area for neutral loci (white area). LOSITAN software was used.