

Genetic component of flammability variation in a Mediterranean shrub

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MOLECULAR ECOLOGY**Genetic component of flammability variation in a
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1 **Genetic component of flammability variation in a Mediterranean shrub**

2 B. Moreira^{1,2}, M. C. Castellanos¹, J. G. Pausas^{1*}

3 ¹ CIDE-CSIC, Ctra. Náquera Km. 4.5, 46113 Montcada, Valencia, Spain

4 ² Current address: CFE – Centre for Functional Ecology, Department of Life Sciences,

5 University of Coimbra, 3001-401 Coimbra, Portugal

6 *Corresponding author; e-mail: juli.g.pausas@uv.es; Fax: (+34) 963424160

7

8 Running title: Genetic component of flammability variation

9

10 **Abstract**

11 Recurrent fires impose a strong selection pressure in many ecosystems worldwide. In such
12 ecosystems, plant flammability is of paramount importance because it enhances population
13 persistence, particularly in non-resprouting species. Indeed, there is evidence of phenotypic
14 divergence of flammability under different fire regimes. Our general hypothesis is that
15 flammability-enhancing traits are adaptive; here we test whether they have a genetic
16 component. To test this hypothesis we used the post-fire obligate seeder *Ulex parviflorus* from
17 sites historically exposed to different fire recurrence. We associated molecular variation in
18 potentially adaptive loci detected with a genomic scan (using AFLP markers) to individual
19 phenotypic variability in flammability across fire regimes. We found that at least 42% of the
20 phenotypic variation in flammability was explained by the genetic divergence in a subset of
21 AFLP loci. In spite of generalised gene flow, the genetic variability was structured by
22 differences in fire recurrence. Our results provide the first field evidence supporting that traits
23 enhancing plant flammability have a genetic component and thus can be responding to natural

2

24 selection driven by fire. These results highlight the importance of flammability as an adaptive
25 trait in fire-prone ecosystems.

26

27 **Key words:** AFLP genome scan, fire regime, phenotype-loci associations, plant flammability,
28 obligate seeder, *Ulex parviflorus*

For Review Only

29 **Introduction**

30 Wildfires are an ancient and widespread phenomenon on the Earth (Pausas & Keeley 2009;
31 Pausas & Ribeiro 2013) and have played a significant role in the distribution of vegetation
32 (Bond *et al.* 2005; Keeley & Rundel 2005), in the evolution of different plant lineages
33 (Pausas & Verdú 2005; Crisp *et al.* 2011; He *et al.* 2011, 2012) and in the structure of plant
34 communities (Pausas *et al.* 2004; Verdú & Pausas 2007). While there is an emerging view
35 suggesting that fires can be strong agents of selection of plant traits (Keeley *et al.* 2011;
36 Pausas & Schwilk 2012), few population-level studies of trait variability in response to fire
37 have been reported.

38 Recently, a few studies have provided firm evidence for fire-driven selection on plant traits
39 by focusing on trait variation across natural populations with contrasting fire regimes.
40 Gómez-González *et al.* (2011) showed adaptive changes in seed traits under different fire
41 regimes of an annual species living in an area where fire is a novel disturbance. In ecosystems
42 where fire is ubiquitous (e.g., Mediterranean communities), a phenotypic effect of variation in
43 fire recurrence can also be detected. For instance, there is evidence of higher serotiny levels
44 (i.e., higher proportion of serotinous cones or longer cone retention for seed release in
45 response to fire) in pine populations living in areas under crown-fires, compared to those
46 growing in areas that rarely burn (Gauthier *et al.* 1996; Radeloff *et al.* 2004; Goubitz *et al.*
47 2004; see the review by Hernandez-Serrano *et al.* 2013). Similarly, Pausas *et al.* (2012)
48 showed that individuals of the Mediterranean shrub species *Ulex parviflorus* from localities
49 with a history of high fire recurrence are, on average, more flammable than those growing in
50 sites with no recent fires. These studies strongly suggest that phenotypic variation in fire-
51 related traits can be the consequence of different fire regimes across the landscape. However,
52 there are few studies analysing whether the intraspecific phenotypic variation observed in

53 these fire-related traits is genetically determined (Pausas & Schwilk 2012) and the scarce
54 evidence is mainly related to regeneration traits (Gómez-González *et al.* 2011; Parchman *et*
55 *al.* 2012; Budde *et al.* 2014).

56 A set of traits extremely relevant in fire-prone ecosystems are vegetative traits that enhance
57 plant flammability. Being flammable can be beneficial, particularly in species from fire-prone
58 communities that do not resprout after a fire but instead rely on the formation of persistent
59 seedbanks and on fire-stimulated germination for recruitment (i.e., post-fire seeders). Adults
60 of these species die when affected by fire but the germination of their soil-stored seeds is
61 enhanced by the high temperatures produced during the fire (Keeley & Fotheringham 2000;
62 Moreira *et al.* 2010). In fact, flammability increases both the probability of ignition and the
63 heat released to the soil (Pausas & Moreira 2012). In addition, being highly flammable can
64 increase the mortality of neighbours and therefore improve the chances of seedling
65 recruitment by lowering competition for resources after the fire (Bond & Midgley 1995).
66 Flammability is thus an important biological attribute that can be expected to be under natural
67 selection (Schwilk & Kerr 2002; Pausas *et al.* 2012).

68 Flammability-enhancing traits can be of different types, including whole-plant structural
69 traits such as high surface-to-volume ratio and retention of dead biomass (Papió & Trabaud
70 1990, 1991; van Wilgen *et al.* 1990; Schwilk 2003), and tissue-level chemical traits, such as
71 high cellulose : lignin ratio and high levels of flammable compounds (Philpot 1970; Rundel
72 1981; Dimitrakopoulos *et al.* 2001; Alessio *et al.* 2008). Because of this complexity, studying
73 the genetic basis of flammability might be difficult, particularly when dealing with non-model
74 perennial species in natural conditions for which information on neither genealogical
75 relationships among individuals nor genomic resources are available. A useful and time-
76 effective approach in these cases is to implement a genome-wide scan that can highlight

77 polymorphisms with a potentially adaptive basis, in combination with individual-level
78 information on the phenotypic variation of interest. This is analogous to linkage
79 disequilibrium or association mapping, but without requiring previous genomic knowledge; it
80 can be performed with genome-wide scans using anonymous markers (such as AFLP loci;
81 e.g., Herrera & Bazaga 2009) or with multiplexed genome-wide sequencing using recent
82 techniques for non-model species (e.g., Gompert *et al.* 2010; Cosart *et al.* 2011; Parchman *et*
83 *al.* 2012; Budde *et al.* 2014). Linking individual variation in molecular markers and
84 phenotypic trait values is possibly the most informative method available to obtain evidence
85 for the adaptive value of outlier loci (Luikart *et al.* 2003; Pannell & Fields 2014).

86 Our hypothesis is that flammability-enhancing traits in post-fire seeders vary adaptively in
87 response to different fire regimes, and thus we expect the phenotypic variation in these traits
88 to have a genetic component. To test this hypothesis we used *Ulex parviflorus*, a typical post-
89 fire seeder living in Mediterranean ecosystems. We studied the patterns of molecular variation
90 in AFLP loci (i.e. specific genomic regions detected with the Amplified Fragment Length
91 Polymorphism technique) among individual plants, from sites historically exposed to different
92 fire regimes, and related such variation with the phenotypic variability in flammability.

93

94 **Materials and Methods**

95 Study species

96 *Ulex parviflorus* Pourr. (Mediterranean gorse, Fabaceae) is a thorny perennial shrub
97 (commonly lacking true leaves in the adult stage) that grows up to *ca.* 2 m. Flowers are
98 hermaphrodite; they open in winter and are pollinated by bees (Herrera 1987). Fruits are dry
99 legumes with explosive dehiscence (June-July) and contain one to four seeds with an
100 elaiosome.

101 This species is very common and widespread along the coast of the western
102 Mediterranean Basin, with continuous distribution along the Iberian coast, from southern
103 Portugal to southern France. As it lacks the ability to resprout (it is an obligate seeder), *U.*
104 *parviflorus* relies entirely on seedling recruitment for post-fire persistence (Paula *et al.* 2009).
105 Seed production is high and seeds have a hard, water-impermeable seed coat that prevents
106 germination and allows most seeds to be stored in a persistent soil seedbank until the
107 dormancy is relieved. Germination can take place in open sites such as old fields (Baeza *et al.*
108 2011), but recruitment is mainly restricted to post-fire conditions, because seed dormancy
109 relief and stimulated germination is mostly triggered by the high temperatures reached in the
110 soil during a fire (Moreira *et al.* 2010; Moreira & Pausas 2012). Indeed, this species has
111 flammability-enhancing traits that increase the probability of fire and favour high
112 temperatures towards the soil (Pausas *et al.* 2012; Pausas & Moreira 2012).

113

114 Study sites and sampling

115 This study was conducted in the eastern Iberian Peninsula (Valencia, Spain), a typical
116 Mediterranean climate area (Pausas 2004). In this region, *U. parviflorus* is very common and
117 continuously distributed from the coast up to about 900 m of altitude. Individuals were
118 sampled at sites with contrasting fire regimes. After a careful field survey assisted by the local
119 government forest fire database, we selected two sites within high-fire recurrence areas and
120 two sites within unburned areas where *U. parviflorus* is abundant (hereafter HiFi and NoFi
121 sites, respectively; Table 1; see Pausas *et al.* (2012) for further details on site selection and
122 characteristics). NoFi individuals grow in old-fields where the recruitment of recent
123 generations is independent of fire (old-field colonization). In contrast, HiFi sites are the
124 product of recurrent fires, and the recruitment of most individuals has been mediated by fire

125 (post-fire regeneration) for at least three generations. That is, while in HiFi sites there has
126 been fire-related selection (e.g., for fire-resistant seeds) associated to the previous recruitment
127 events, this is not the case for NoFi sites. Thus, these sampling sites span the variability of
128 habitats where this species is dominant (recurrently burned areas and abandoned fields) and of
129 fire regimes in the region. All sites were shrublands growing on calcareous bedrock. To assess
130 the relation between environmental conditions and fire regime, at each site we collected five
131 soil samples and climatic information from a local climatic atlas. The analysis of this data
132 showed that the different fire regimes are not related to differences in environmental
133 conditions (Table 1). For instance, mean soil pH in the different sites ranges from 7.7 to 8.0
134 and does not show a relationship with fire regime (Table 1). In addition, the variability in
135 altitude and climate within NoFi sites is larger than between fire regimes (Table 1); that is,
136 sites at the highest and lowest altitudes have both been regenerated by old-field colonization
137 (NoFi) and are the ones with the lowest flammability (Pausas *et al.* 2012). Furthermore, the
138 four sites do not exhibit geographical aggregation following the different fire regimes; one of
139 the NoFi sites is *ca.* 110–115 km from the other three, while the other three sites are 12–28
140 km apart (Table 1, Tables S1, S2, Supporting information). In sum, biogeographical
141 differences should not bias the differences between NoFi and HiFi.

142 In each site we selected and geo-referenced 40–46 mature individuals (a total of 169
143 individuals), separated by at least 5 m from each other. The final distances between
144 individuals depended on the density of mature non-senescent individuals and the landscape
145 conditions at each site (e.g., old-field size, topography), with maximum distance ranging from
146 130 to 538 m, median pairwise distance ranging from 35 to 173 m and median distance to the
147 nearest neighbour ranging from 5.7 to 14.7 m (Table S1, Supporting information). In June of

148 2010 we collected a terminal twig (last growing season tissue) from each individual and dried
149 and preserved them in silica gel until DNA extraction.

150

151 Flammability measurements

152 The same individuals that were sampled for genetic analysis had been previously
153 characterized for flammability by measuring plant structural traits and performing
154 flammability experiments in live twigs using an epiradiator (see Pausas *et al.* (2012) for
155 details). In brief, flammability variables analysed at twig level were: time to ignition (i.e. time
156 to initiate a flame; s), mass loss rate (fresh biomass consumed divided by the flame duration;
157 mg s^{-1}), heat released during combustion (area under the temperature–time curve during the
158 flame duration divided by the sample fresh biomass; $^{\circ}\text{C s g}^{-1}$), and maximum temperature
159 ($^{\circ}\text{C}$) reached by the flame. In addition, for each individual, the proportion of dry biomass of
160 the different fuel classes (%) and the plant bulk density (i.e. plant dry biomass per volume; g
161 cm^{-3}) were estimated. The results of this study showed that plants from HiFi sites ignited
162 earlier, burned more slowly, released more heat and had higher bulk density than plants from
163 NoFi sites (Pausas *et al.* 2012; Pausas & Moreira 2012).

164

165 Genomic extraction and AFLP scoring

166 For each individual we extracted genomic DNA from *ca.* 50 mg of dried plant
167 material, previously powdered using two stainless steel beads on a RETSCH MM 400 mixer
168 mill. The extraction was performed using the Speedtools plant DNA extraction kit (Biotools,
169 Madrid, Spain), with small modifications to the manufacturer's protocol to optimize the
170 extraction for this highly lignified species. DNA quantity and quality was assessed by
171 NanoDrop™ 1000 and by running electrophoreses of aliquots of the genomic DNA extracted

172 on a 0.9% agarose gel to confirm that the DNA consisted of a single, intact and high
173 molecular weight band.

174 The AFLP analysis was performed using the technique by Vos et al. (1995) and
175 following the recommendations of Meudt & Clarke (2007) (see a detailed protocol in
176 the Supporting information Protocol S1). Restriction-ligation was performed using
177 EcoRI/MseI endonuclease mixture and double-stranded adaptors. We first performed
178 screening of selective primer combinations (*ca.* 50 EcoRI+3/MseI+3 combinations) on a
179 subset of 12 individuals from the different sites and selected the nine primer combinations
180 with the highest quality profiles (Table S3, Supporting information).

181 A total of 169 plants were fingerprinted using the nine primer combinations. Selective
182 amplification products were poolplexed and detected using an ABI PRISM 3730 automated
183 DNA sequencer (Applied Biosystems). Band presence/absence was scored manually for each
184 individual plant through the visualization of the electropherograms with GeneMarker v. 1.85
185 software (Softgenetics, State College, USA). For each marker, the scoring threshold was
186 determined by contrasting the corresponding peaks against the background, after
187 normalization of the profiles. All scoring was carried out by the same person, and information
188 on phenotypic characteristics of individual plants was unknown at the time of scoring. Only
189 fragments within the range of 70-550 bp were considered. Only polymorphic peaks that
190 overlapped homogeneously when all samples were superimposed were accepted for further
191 analysis. Finally, AFLP loci that were present in <1% or > 99% of the individuals were
192 excluded from the final data set. The exact number of loci and individuals considered
193 depended on the specific analysis (Table S4, Supporting information).

194 For a subset of 15 plants (8% of the total sample size), DNA extraction and AFLP
195 analyses were performed twice to determine error rates, including both technical and human

196 errors (Bonin *et al.* 2004; Pompanon *et al.* 2005). Error was estimated as the ratio of the total
197 number of AFLP loci with contradictory scores on those two independent analyses to the
198 product of the total number of individuals by the total number of AFLP loci scored. These rates
199 varied among loci and primer combinations (Table S3, Supporting information), and averaged
200 2.0% (± 2.4 SD) across all loci. Most errors detected were corrected previous to the analysis,
201 and thus these error rates are probably an upper limit.

202

203 Within- and among-site genetic variation

204 To estimate within-site levels of diversity we assumed that **populations** of this
205 outcrossing species were in Hardy-Weinberg equilibrium. We calculated Nei's gene diversity
206 (H_j) and the proportion of polymorphic loci (PLP) for each site in AFLPsurv (Vekemans *et al.*
207 2002). In addition, we report band richness estimates, an analogue of allelic richness,
208 calculated with the rarefaction approach of Coart *et al.* (2005) implemented in AFLPdiv for
209 30 individuals.

210 The level of genetic differentiation among sites was estimated in three different ways.
211 First, we used Genalex v.6.5 (Peakall & Smouse 2006, 2012) to estimate overall Φ_{PT} values as
212 well as paired Φ_{PT} values between sites. For this, the between- and within-group variances
213 were calculated via AMOVA of genetic distances between sample **multilocus genotypes**. Φ_{PT}
214 is an analogue of F_{ST} for binary data. The significance of the model and of each estimate was
215 tested through 9999 permutations over the whole data set. Second, we estimated overall and
216 paired F_{ST} values based on allele frequencies as implemented in AFLPsurv (Vekemans *et al.*
217 2002), using the Bayesian method with non-uniform prior distribution of Zhivotovsky (1999).
218 The significance of the overall estimate was based on 10000 random permutations of
219 individuals among sites. Finally, we computed D following equation 11 in Jost (2008) and

220 using the estimates of heterozygosity provided in the output of AFLPsurv (using H_t as H_T , and
221 H_w as H_S). D is a measure of differentiation estimated as the proportion of the total diversity
222 that is contained in the average locality, and is independent of within-locality heterozygosity.

223

224 Phenotypic characterization of flammability

225 Flammability variables (see above) were summarized into two orthogonal axes of
226 variation using a principal components analysis (PCA) including all individuals (flamPC1 and
227 flamPC2 hereafter). Because the flammability parameters measured at twig level were
228 significantly related to twig moisture at the time of testing (Pausas *et al.* 2012), the PCA was
229 performed with the residuals of the flammability variable regressed against moisture. This
230 procedure accounts for the effect of the flammability variable once it has been corrected for
231 moisture. The PCA was performed with the basic *stats* package in R (R core team 2013) with
232 variables transformed to achieve normality, when required.

233 FlamPC1 explained 58.0% of the variance in flammability and was mostly a gradient
234 of time to ignition and mass loss rate (negative) and heat release (positive). FlamPC2
235 explained 21.5% of the variance and was highly associated to bulk density (Table S5, Fig. S1,
236 Supporting information). That is, flamPC1 was more related to small scale (i.e., twig)
237 flammability while flamPC2 reflected flammability associated to whole-plant structure. We
238 thus considered that these two PC axes successfully summarize the phenotypic variability in
239 flammability across individuals.

240

241 Genetic and phenotypic association

242 To evaluate possible links between individual variation in DNA markers and
243 phenotypic traits we searched for significant associations across individuals between AFLP

244 loci (presence/absence) and flammability (flamPC axes) following the approach by Herrera &
245 Bazaga (2009). For this analysis, AFLP loci that were present in < 5% or > 95% of the
246 individuals were discarded because parameter estimates might be excessively influenced by
247 outlying data, producing spurious results without ecological meaning. Thus the final number
248 of AFLP loci considered for the genetic-phenotypic association was 226 (Table S4,
249 Supporting information). Unless otherwise noted, analyses were run in the *stats* package in R.
250 For each locus, we performed two GLM regressions with binomial error distribution using
251 band presence/absence as the dependent variable and each plant flammability indicator
252 (flamPC1 or flamPC2) as the independent variable. The significance of these regressions was
253 obtained after accounting for the possibility of obtaining false significant regressions (i.e.
254 committing type I errors) due to the large number of comparisons performed, using the q-
255 value method for estimation of false discovery rates (Storey & Tibshirani 2003; using *qvalue*
256 package in R, Dabney & Storey 2013). We ascertained the q-value threshold leading to an
257 expectation of less than one falsely significant regression. That is, we used the largest q-value
258 for which the resulting multiplication by the number of regressions accepted as significant
259 (i.e., regressions with q-value lower than the threshold) was lower than one (Herrera &
260 Bazaga 2009). To further ensure that the set of loci significantly associated to flamPC1 and
261 flamPC2 are not the product of chance alone, we randomly permuted the AFLP
262 presence/absence matrix and performed the regressions with flammability variables as above.
263 Permutations were performed within locus (i.e., across individuals) for the complete data set
264 (including all localities) and repeated 100 times with the help of the *picante* package in R
265 (Kembel *et al.* 2010). The number of significant regressions after the q-value adjustment was
266 compared with the observed number of significant regressions in the real dataset.

267 To increase confidence in the selection of AFLP loci related to flammability, all
268 significant regressions (after correction) were reanalysed using the Markov Chain Monte
269 Carlo approach for logistic regression implemented in the *MCMCpack* package (Martin *et al.*
270 2011). We used 50 000 burn-in iterations, 500 000 Metropolis iterations and a thinning
271 interval of 1000. The AFLP loci that were significant for any of the two flammability
272 variables and in both analyses can be considered as putative “adaptive loci” (*sensu* Herrera &
273 Bazaga (2009)).

274 To estimate the amount of phenotypic variance in flammability explained by these
275 significant loci, for each flammability axis (flamPC1 and flamPC2) we fitted a multiple linear
276 regression against these loci. Finally, to summarize and graphically display the relationship
277 between individual flammability and genotypic variability, each flammability variable was
278 related to a principal coordinate analysis (PCoA) performed with the corresponding set of
279 significant loci. This analysis also allowed us to include site as a random factor and to
280 consider possible spatial autocorrelation. For this, we constructed a matrix of pairwise linear
281 genetic distances between individuals using these loci and performed a PCoA, based on the
282 covariance matrix, in Genalex. We used the two main orthogonal axes of variation (lociPCo1,
283 lociPCo2) to represent the genetic distance between individuals. We then fitted, for each
284 flammability indicator (flamPC1 and flamPC2), a mixed effect model to individual plant data,
285 using flammability as the response variable, the genetic distance (lociPCo1 and lociPCo2) as
286 independent variables, and site as a random factor; significance was tested using a likelihood
287 ratio test (LRT). Mixed models were fitted with the *nlme* package in R (Pinheiro *et al.* 2013).
288 To evaluate the possible effect of the geographic distance in this regression, we compute the
289 spatial autocorrelation of the residuals (Moran’s I statistic) using the *spdep* and *pgirmess*
290 packages of R (Giraudeau 2013); low autocorrelation of the residuals would imply that the

291 regressions are not affected by spatial effects (Diniz-Filho *et al.* 2003). Differences in the axes
292 values between fire regimes for both flammability and genetic axes were also evaluated using
293 a mixed model with site as a random factor and tested with a LRT.

294

295 F_{ST}-outlier loci method

296 Our estimation of putative adaptive loci was based on their relation with flammability.
297 To further support that there is a genetic basis to the phenotypic differences observed in the
298 field, we additionally searched for outlier loci using a population genomic approach (i.e. a
299 phenotype-independent method) as implemented in the software BayeScan 2.1 (Foll &
300 Gaggiotti 2008). This method is based on differentiation between populations, highlighting
301 loci with exceptional genetic differentiation when compared to the neutral expectation. A
302 global analysis based on the AFLP markers was run for the two fire regime categories (a two-
303 deme model: HiFi and NoFi). We pooled sites within the same fire regime because our
304 purpose was to detect allelic variation specifically related to fire. BayeScan automatically
305 tuned model parameters using short pilot runs (20 pilot runs, length 5000). The default chain
306 parameters worked well for our data, so the sample size was 5000 with a thinning interval of
307 10. We set the prior odds to the neutral model to 1, and set the uncertainty for the inbreeding
308 coefficient (F_{is}) prior to vary uniformly between 0.3 and 0.7. This is because our study plant
309 has a mixed mating system, as it is self-compatible but pollinated by effective pollinators
310 (large bees) which are highly likely to perform cross-pollination.

311

312 **Results**

313 A total of 376 polymorphic loci were scored from the nine primer combinations for
314 each of 169 individual plants in the four study sites. A final dataset excluding loci where the

315 most frequent allele had a frequency $\geq 99\%$ comprised 329 AFLP loci and yielded within-site
316 genetic diversity values (H_j , PLP and Br) that were similar among sites (Table 2). Estimates
317 of among-sites differentiation showed low genetic differences in spite of geographical
318 distances. The vast majority of the molecular variance occurred within sites (92%), leaving
319 the remaining 8% to variance among sites (AMOVA, $P < 0.001$, overall $\Phi_{PT} = 0.078$). This is
320 confirmed by the other two estimates, overall $F_{ST} = 0.05$ and $D = 0.02$. The site located furthest
321 from the other three, Ares del Maestrat (Table 1 and Table S2, Supporting information), was
322 also the most genetically distant, as expected. However, paired F_{ST} and Φ_{PT} values between
323 this site and the others were still low ($F_{ST} = 0.06-0.07$ and $\Phi_{PT} = 0.10-0.11$, see Tables S6 and
324 S7, Supporting information). The remaining sites had paired F_{ST} values between 0.02 and
325 0.04, and paired Φ_{PT} values between 0.04 and 0.06. In addition, grouping sites with the same
326 fire regime for the analysis (HiFi and NoFi sites) and comparing their F_{ST} estimates also
327 yielded low differentiation ($F_{ST} = 0.02$). These results suggest that geographical separation
328 among our study sites does not prevent gene flow among them. Spatial effects do occur,
329 possibly due to isolation by distance, but our results imply that all sites are interconnected, as
330 expected by the high abundance and the widespread distribution of this species in the study
331 region. Therefore, we pooled all individuals from the four sites for the remaining analyses of
332 genetic and phenotypic association.

333 A total of 16 loci were significantly related to flamPC1 and 13 to flamPC2 (Table 3),
334 with both positive and negative relationships between flammability and the presence of a
335 locus (Fig. 1). When we computed the significance of the flammability axes against the AFLP
336 loci in the randomly permuted data matrices, we obtained between 0 and 5 significant
337 regressions, further suggesting that our results cannot be explained by chance. The multiple
338 regression analysis of flamPC1 in relation to the presence/absence of the 16 loci was highly

339 significant ($F_{16,142} = 8.215$, $P < 0.0001$, $R^2 = 0.48$, adjusted $R^2 = 0.42$), accounting for 42% of
340 individual variance in flammability. For flamPC2, the relationship with the 13 corresponding
341 loci was also highly significant and accounted for 30% of the variance ($F_{13,145} = 6.315$, $P <$
342 0.0001 , $R^2 = 0.36$, adjusted $R^2 = 0.30$). Excluding the few significant loci that are exclusive
343 from the most distant site (1 out of 16 for flamPC1 and 4 out of 13 for flamPC2; Tables S8
344 and S9, Supporting information) barely influenced the multiple regression analysis (adjusted
345 $R^2 = 0.42$ for flamPC1 and adjusted $R^2 = 0.28$ for flamPC2).

346 The two axes of the genetic PCoA performed with the 16 loci significantly related to
347 flamPC1 explained 28.58% (lociPCo1) and 19.14% (lociPCo2) of the molecular variance. For
348 the PCoA from the 13 loci significantly related to flamPC2, the axes explained 31.04%
349 (lociPCo1) and 20.59% (lociPCo2) of the variance. Plant flammability (both flamPC1 and
350 flamPC2) were significantly related to the genetic variation summarized in the PCoA scores
351 (Table 4, Fig. 2). While the two flammability variables showed a strong spatial autocorrelation
352 (Moran's I for flamPC1 = 0.15 and for flamPC2 = 0.20, $p < 0.0001$), the residuals of the
353 regression were not spatially autocorrelated for any of the two models (Moran's I = -0.01, $p >$
354 0.65). The two flammability axes (flamPC1 and flamPC2) as well as the genetic axis from
355 potentially adaptive loci in flamPC1 (lociPCo1) were significantly different between fire
356 regimes (different colours in Fig. 2; LRT: flamPC1, $p = 0.004$; flamPC2, $p = 0.029$; lociPCo1,
357 $p = 0.047$). The latter value suggests that at least part of the genetic variability is structured by
358 fire regime.

359 The phenotypic-independent outlier detection analysis showed that four AFLP loci out
360 of the 220 analysed (Table S4, Supporting information) were outlier loci. All of these four loci
361 were already pointed out in the previous analysis as being related to one or both flammability
362 variables (Table 3). Figure 3 shows an example of one such relationship, where the allelic

363 frequencies of locus P5-216 in the four study sites are strongly correlated with flammability
364 (see also Fig. S2 in Supporting Information for a plot with the relation between this marker
365 and geographical distance between sites). We confirmed that none of the four loci were
366 simply associated to a single site by performing two independent runs in BayeScan, but
367 limited to within HiFi and NoFi sites respectively (i.e., two sites in each); none of the four
368 loci detected in the overall analysis appeared in these two independent runs (results not
369 shown).

370

371 Discussion

372 Recurrent fires exert a strong evolutionary pressure in post-fire obligate seeders
373 because fire kills established individual plants and population persistence is attained by a
374 profuse recruitment from soil or canopy seedbanks (Keeley *et al.* 2011; Moreira & Pausas
375 2012). In these species, being flammable provides fitness benefits because fires break seed
376 dormancy and open up microsites for recruitment (Bond & Midgley 1995; Schwilk 2003;
377 Moreira *et al.* 2010; Pausas *et al.* 2012; Pausas & Moreira 2012). Here we showed that, for
378 the post-fire obligate seeder *Ulex parviflorus*, an important part of the phenotypic variability
379 in plant flammability has a genetic component (at least 42%), and that the genetic variability
380 in putative adaptive loci is structured by differences in fire recurrence. These results, together
381 with the concurrent phenotypic divergence in this species, associated to different fire regimes
382 (Pausas *et al.* 2012), further support the adaptive nature of flammability.

383 The phenotypic-oriented approach for finding adaptive molecular variation, combining
384 a whole-genome scan analysis with the use of individual phenotypic data, allowed us to
385 suggest that fire might drive changes in allelic frequencies across natural sites and that such

386 variability is associated with particular flammability phenotypes. It is possible that some of
387 the genetic variation could be associated to traits that covary with flammability in response to
388 other factors (e.g., environmental characteristics, geographical distance). However, this is
389 improbable because fire regimes do not vary in parallel with either environmental conditions
390 or with the geographical distribution of the sites (Table 1). Despite we used a relatively large
391 number of individuals, one caveat of this study is that it is based on a limited number of sites
392 (two HiFi and two NoFi sites). We limited the analysis to the sites that we were confident of
393 fire history and that individual level phenotypic variation has been carefully studied.

394 The relatively large number of AFLP loci correlated with flammability (26 loci, Table
395 3) could be explained by the complexity of this compound trait. In fact, flammability can be
396 determined by very different plant attributes such as whole-plant structure, complex tissue
397 composition including organic and inorganic compounds, and water retention strategies (van
398 Wilgen *et al.* 1990; Schwilk 2003; Alessio *et al.* 2008). The potential polygenic nature of
399 flammability probably reflects the high natural variation observed in distinct flammability-
400 enhancing traits, such as time-to-ignition or heat release, detectable even among nearby
401 individuals of *U. parviflorus* (Pausas *et al.* 2012). In addition to relating flammability
402 phenotypes directly to molecular variation, we also used an F_{ST} -based outlier loci detection
403 method to yield an independent confirmation of the presence of loci with exceptional
404 differentiation between fire regimes (see e.g. Keller *et al.* 2012 for a similar combined
405 approach). Four of the loci associated to flammability were also highlighted as outliers with
406 this approach, further supporting the potential existence of genomic regions under selection
407 by fire.

408 The divergence in flammability in *U. parviflorus*, associated with differences in fire
409 recurrence, occurs in spite of the generalized gene flow that keeps geographically distant

410 localities interconnected. This partly explains why there is no evidence of reduction in genetic
411 diversity after recurrent fires in our study, as well as in previous ones (Ayre *et al.* 2009). In
412 addition, the spatial heterogeneity of fires and the fact that seedbanks act as a genetic
413 reservoir, are likely to buffer populations of seeder species against genetic erosion (Bahulikar
414 *et al.* 2004; Ayre *et al.* 2009). This fire-induced variability is also observed in phenotypic
415 traits important for persistence in fire-prone ecosystems such as regeneration traits (Moreira *et*
416 *al.* 2012).

417 Our association study used 226 AFLP loci, which necessarily cover only a fraction of
418 the genome of *U. parviflorus*. It is however remarkable that with this low coverage and
419 relatively low number of individuals and sites, the method allowed us to detect a number of
420 loci that together explain at least 42% of the phenotypic variance in the sample individuals.
421 The AFLP technique, even with its limitations, provided a time and cost-effective approach to
422 understanding the genetic architecture of flammability-enhancing traits. Although AFLP loci
423 might not necessarily be linked to functional genes they allow the preliminary detection of a
424 genetic basis for complex traits. However, with sequencing techniques that provide wide
425 genome coverage to generate population genomic data advancing vertiginously and prices
426 plummeting, the next step will be to use high numbers of markers to further study quantitative
427 genetic parameters for fire-related traits.

428 Evidence of adaptive divergence driven by fire is currently growing (Pausas &
429 Schwilk 2012), although most studies focus on a single trait, serotiny. Pine serotiny varies
430 with fire regime (Hernández-Serrano *et al.* 2013) and there is evidence that variability in
431 common garden trials follows closely that of parental stands (Kuser & Ledig 1987; Ledig *et*
432 *al.* 2013). In addition, recent genomic scans have detected a strong genetic signal for this trait
433 (Parchman *et al.* 2012; Budde *et al.* 2014). The only other evidence of fire-driven adaptive

434 divergence comes from an annual plant whose seed traits are heritable and vary predictably
435 under different fire regimes (Gómez-González *et al.* 2011). Thus our results provide the first
436 field evidence supporting that traits enhancing plant flammability may be responding to
437 natural selection driven by fire. This conclusion agrees with recent studies on the crucial role
438 of flammability in key moments of the evolutionary history of plants (Bond & Scott 2010)
439 and specifically in the diversification of plant lineages subject to strong fire pressures
440 (Schwilk & Ackerly 2001; He *et al.* 2011, 2012). Altogether, these various studies point
441 towards the key role of flammability in plant evolution, a characteristic that has often been
442 neglected in the evolutionary literature.

443

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454

455 **Data accessibility**

456 AFLP and phenotypic data: DRYAD entry doi: xx.xxxx/dryad.xxxx

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612

613 Author contributions

614 JGP designed the study; BM performed the sampling; BM and MCC performed the molecular
615 analyses; BM, MCC and JGP analysed the data and wrote the first version of the manuscript;
616 MCC and JGP wrote the final version.

617

618 **Figure legends**

619

620 Figure 1. Relationship between plant flammability (flamPC1, flamPC2) and AFLP scores (1:
621 AFLP locus presence; 0: AFLP locus absence) for loci P5-216 (left) and P3-289 (right) across
622 *Ulex parviflorus* individuals (HiFi in closed triangles, NoFi in open circles). Closed symbols
623 are slightly moved down for clarity. Statistics are reported in Table 3.

624

625 Figure 2. Relationship between individual *Ulex parviflorus* flammability and genotypic
626 distance based on potentially adaptive AFLP loci (lociPCo1 score), for flamPC1 (left) and
627 flamPC2 (right). The significant ($p < 0.001$) regression lines are also plotted. The two
628 flammability axes (flamPC1 and flamPC2) and the genetic axis from putative adaptive loci in
629 flamPC1 (lociPCo1, left figure) were significantly different between fire regimes (HiFi in
630 closed triangles, NoFi in open circles; LRT: flamPC1, $p = 0.004$; flamPC2, $p = 0.029$;
631 lociPCo1, $p = 0.047$).

632

633 Figure 3. Relationship between allelic frequency of locus P5-216 and average flammability
634 (flamPC1 score) for the four study sites. Sites are Ares del Maestrat and Cheste (NoFi; in
635 open circles), Sot de Chera and Chiva (HiFi, in closed triangles).

636

637 **Tables**

638

639 Table 1. General characteristics of each site, including location, fire regime (NoFi sites were
 640 in unburned areas, while HiFi sites had high fire recurrence), fire years (during the period
 641 1978-2010), soil pH, mean annual temperature, annual precipitation (Prec.), altitude (Alt.),
 642 and distance to the nearest site (Dist.). Soil pH values are not significantly different between fire
 643 regimes ($p=0.42$; mixed model with site as random factor).

Location	Fire regime	Fire years	Soil pH	T (°C)	Alt. (masl)	Prec (mm)	Dist. (km)
Ares del Maestrat	NoFi	None	7.8 ± 0.10	14.4	820	760	109.8
Cheste	NoFi	None	8.0 ± 0.08	17.7	170	422	16.2
Sot de Chera	HiFi	1978,1986,1994	7.8 ± 0.06	14.2	775	600	12.9
Chiva	HiFi	1990,1994,2000	7.7 ± 0.18	15.0	800	553	12.9

644

645

646 Table 2. Genetic diversity estimates for each site based on 329 AFLP loci. N= number of
647 individuals sampled (genotyped and phenotyped) individuals, PLP= proportion of
648 polymorphic loci, H_j = unbiased Nei's gene diversity (\pm SE), Br= band richness.

Location	N	PLP	H_j (Mean \pm SE)	Br
Ares del Maestrat	39	62.6	0.225 \pm 0.0102	1.76
Cheste	40	64.1	0.225 \pm 0.0102	1.74
Sot de Chera	46	62.9	0.216 \pm 0.0099	1.73
Chiva	44	60.8	0.217 \pm 0.0100	1.74

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649 Table 3 – Results of the independent logistic regressions (GLM) across individuals of AFLP
 650 loci presence/absence against two condensed flammability variables (flamPC1 and flamPC2).
 651 Only the results for the loci with statistically significant relationships after correction for false
 652 discovery rates are shown (16 for flamPC1 and 13 for flamPC2). See supporting information
 653 Tables S8 and S9 for the logistic regression obtained with a Bayesian approach. AFLP locus
 654 names refer to the primer combination (Table S3, Supporting information) and the size of the
 655 fragment (in bp). AFLP loci with an asterisk (*) are outlier loci detected by the phenotype-
 656 independent method in BayeScan. Coef.: linear regression coefficient; se: standard error; p: p-
 657 value.

AFLP locus	flamPC1			AFLP locus	flamPC2		
	Coef.	se	p		Coef.	se	p
P2-293	0.41	0.13	0.001	P2-95	-0.60	0.20	0.002
P2-395	0.32	0.11	0.003	P2-222	-0.90	0.31	0.004
P3-195	0.45	0.13	0.001	P2-381	1.19	0.35	0.001
P3-314	-0.47	0.15	0.002	P3-289	-0.62	0.19	0.002
P1-199	-0.33	0.12	0.004	P1-284	-0.55	0.21	0.005
P5-208	-0.54	0.17	0.001	P1-289*	-1.86	0.39	<0.001
P5-216*	0.31	0.11	0.005	P6-161*	1.15	0.34	<0.001
P5-425	-0.59	0.16	<0.001	P6-239	-0.72	0.26	0.003
P6-161*	0.64	0.17	<0.001	P4-344	0.90	0.30	0.001
P4-189	-0.47	0.14	<0.001	P8-137	-1.21	0.38	<0.001
P4-344	0.56	0.16	<0.001	P8-300*	-1.99	0.43	<0.001
P8-179	0.60	0.16	<0.001	P9-151	-0.64	0.20	<0.001
P8-213	0.46	0.16	0.004	P7-365	0.61	0.19	<0.001
P8-228	0.31	0.11	0.005				
P9-151	-0.33	0.11	0.002				

P7-211	0.41	0.16	0.005
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658

For Review Only

659 Table 4 – Summary of the likelihood ratio test (LRT) for the linear mixed-effects model of
 660 genotype (lociPCo1 and lociPCo2) against the observed phenotypic variability in
 661 flammability (flamPC1 and flamPC2) in *Ulex parviflorus*. AIC: Akaike information criterion.

	flamPC1			flamPC2		
	AIC	LRT	p	AIC	LRT	p
null	539.51			396.75		
lociPCo1	517.98	23.53	< 0.0001	393.75	5.00	0.0253
lociPCo2	518.17	1.81	0.1780	383.63	12.12	0.0005

662

Locus P5 216

Locus P3 289





