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Genetic component of flammability variation in a Mediterranean shrub

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Abstract

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

Keywords: AFLP genome scan, fire regime, obligate seeder, phenotype–loci associations, plant flammability, *Ulex parviflorus*

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Introduction

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

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Recently, a few studies have provided firm evidence for fire-driven selection on plant traits by focusing on trait variation across natural populations with contrasting fire regimes. Gómez-González *et al.* (2011) showed adaptive changes in seed traits under different fire regimes of an annual species living in an area where fire is a novel disturbance. In ecosystems where fire is ubiquitous (e.g. Mediterranean communities), a phenotypic effect of variation in fire recurrence can also be detected. For instance, there is evidence of higher serotiny levels (i.e. higher proportion of serotinous cones or longer cone retention for seed release in response to fire) in pine populations living in areas under crown-fires, compared to those growing in areas that rarely burn (Gauthier *et al.* 1996; Goubitz *et al.* 2004; Radeloff *et al.* 2004; see the review by Hernández-Serrano *et al.* 2013). Similarly, Pausas *et al.* (2012) showed that individuals of the Mediterranean shrub species *Ulex parviflorus* from localities with a history of high fire recurrence are, on average, more flammable than those

growing in sites with no recent fires. These studies strongly suggest that phenotypic variation in fire-related traits can be the consequence of different fire regimes across the landscape. However, there are few studies analysing whether the intraspecific phenotypic variation observed in these fire-related traits is genetically determined (Pausas & Schwilk 2012) and the scarce evidence is mainly related to regeneration traits (Gómez-González *et al.* 2011; Parchman *et al.* 2012; Budde *et al.* 2014).

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

Flammability-enhancing traits can be of different types, including whole-plant structural traits, such as high surface-to-volume ratio and retention of dead biomass (Papió & Trabaud 1990, 1991; van Wilgen *et al.* 1990; Schwilk 2003), and tissue-level chemical traits, such as high cellulose/lignin ratio and high levels of flammable compounds (Philpot 1970; Rundel 1981; Dimitrakopoulos & Panov 2001; Alessio *et al.* 2008). Because of this complexity, studying the genetic basis of flammability might be difficult, particularly when dealing with nonmodel perennial species in natural conditions for which information on neither genealogical relationships among individuals nor genomic resources is available. A useful and time-effective approach in these cases is to implement a genome-wide scan that can highlight polymorphisms with a potentially adaptive basis, in combination with individual-level information on the phenotypic variation of interest. This is analogous to linkage disequilibrium or association mapping, but without requiring previous genomic knowledge; it can be performed with genome-wide scans using anonymous markers (such as AFLP loci; e.g. Herrera & Bazaga 2009) or with multiplexed genome-wide sequencing using recent techniques for nonmodel species (e.g. Gompert *et al.* 2010; Cosart *et al.* 2011; Parchman *et al.* 2012; Budde *et al.* 2014). Linking individual variation in molecular markers and phenotypic trait values is possibly the most informative method available to obtain evidence for the adaptive value of outlier loci (Luikart *et al.* 2003; Pannell & Fields 2014).

Our hypothesis is that flammability-enhancing traits in postfire seeders vary adaptively in response to different fire regimes, and thus, we expect the phenotypic variation in these traits to have a genetic component. To test this hypothesis, we used *U. parviflorus*, a typical postfire seeder living in Mediterranean ecosystems. We studied the patterns of molecular variation in AFLP loci (i.e. specific genomic regions detected with the amplified fragment length polymorphism technique) among individual plants, from sites historically exposed to different fire regimes, and related such variation with the phenotypic variability in flammability.

Materials and methods

Study species

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

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

Study sites and sampling

This study was conducted in the eastern Iberian Peninsula (Valencia, Spain), a typical Mediterranean climate area (Pausas 2004). In this region, *U. parviflorus* is very

common and continuously distributed from the coast up to about 900 m of altitude. Individuals were sampled at sites with contrasting fire regimes. After a careful field survey assisted by the local government forest fire database, we selected two sites within high fire recurrence areas and two sites within unburned areas where *U. parviflorus* is abundant (hereafter HiFi and NoFi sites, respectively; Table 1; see Pausas *et al.* (2012) for further details on site selection and characteristics). NoFi individuals grow in old fields where the recruitment of recent generations is independent of fire (old-field colonization). In contrast, HiFi sites are the product of recurrent fires, and the recruitment of most individuals has been mediated by fire (postfire regeneration) for at least three generations. That is, while in HiFi sites there has been fire-related selection (e.g. for fire-resistant seeds) associated with the previous recruitment events, this is not the case for NoFi sites. Thus, these sampling sites span the variability of habitats where this species is dominant (recurrently burned areas and abandoned fields) and of fire regimes in the region. All sites were shrublands growing on calcareous bedrock. To assess the relationship between environmental conditions and fire regime, at each site we collected five soil samples and climatic information from a local climatic atlas. The analysis of this data showed that the different fire regimes are not related to differences in environmental conditions (Table 1). For instance, mean soil pH in the different sites ranges from 7.7 to 8.0 and does not show a relationship with fire regime (Table 1). In addition, the variability in altitude and climate within NoFi sites is larger than between fire regimes (Table 1); that is, sites at the highest and lowest altitudes have both been regenerated by old-field colonization (NoFi) and are the ones with the lowest flammability (Pausas *et al.* 2012). Furthermore, the four sites do not exhibit geographical aggregation following the different fire regimes; one of the NoFi sites is *ca.* 110–115 km from the other three, while the other three sites are 12–28 km apart (Table 1, Tables S1 and S2, Supporting Information). In sum, biogeographical differences should not bias the differences between NoFi and HiFi.

In each site, we selected and georeferenced 40–46 mature individuals (a total of 169 individuals), separated by at least 5 m from each other. The final distances between individuals depended on the density of mature nonsenescent individuals and the landscape conditions at each site (e.g. old-field size, topography), with maximum distance ranging from 130 to 538 m, median pairwise distance ranging from 35 to 173 m and median distance to the nearest neighbour ranging from 5.7 to 14.7 m (Table S1, Supporting Information). In June of 2010, we collected a terminal twig (last growing season tissue) from each individual and dried and preserved them in silica gel until DNA extraction.

Flammability measurements

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

Genomic extraction and AFLP scoring

For each individual, we extracted genomic DNA from *ca.* 50 mg of dried plant material, previously powdered using two stainless steel beads on a RETSCH MM 400

Table 1 General characteristics of each site, including location, fire regime (NoFi sites were in unburned areas, while HiFi sites had high fire recurrence), fire years (during the period 1978–2010), soil pH, mean annual temperature, annual precipitation (Prec.), altitude (Alt.) and distance to the nearest site (Dist.)

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

Soil pH values are not significantly different between fire regimes ($P = 0.42$; mixed model with site as random factor).

mixer mill. The extraction was performed using the Speedtools plant DNA extraction kit (Biotools, Madrid, Spain), with small modifications to the manufacturer's protocol to optimize the extraction for this highly lignified species. DNA quantity and quality was assessed by NanoDrop™ 1000 and by running electrophoreses of aliquots of the genomic DNA extracted on a 0.9% agarose gel to confirm that the DNA consisted of a single, intact and high molecular weight band.

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

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

For a subset of 15 plants (8% of the total sample size), DNA extraction and AFLP analyses were performed twice to determine error rates, including both technical and human errors (Bonin *et al.* 2004; Pompanon *et al.* 2005). Error was estimated as the ratio of the total number of AFLP loci with contradictory scores on those two independent analyses to the product of the total number of individuals by the total number of AFLP loci scored. These rates varied among loci and primer combinations (Table S3, Supporting Information) and averaged 2.0% (±2.4 SD) across all loci. Most errors detected were corrected previous to the analysis, and thus, these error rates are probably an upper limit.

Within- and among-site genetic variation

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

The level of genetic differentiation among sites was estimated in three different ways. First, we used GenAlEx version 6.5 (Peakall & Smouse 2006, 2012) to estimate overall Φ_{PT} values as well as paired Φ_{PT} values between sites. For this, the between- and within-group variances were calculated via AMOVA of genetic distances between sample multilocus genotypes. Φ_{PT} is an analogue of F_{ST} for binary data. The significance of the model and of each estimate was tested through 9999 permutations over the whole data set. Second, we estimated overall and paired F_{ST} values based on allele frequencies as implemented in AFLPsurv (Vekemans *et al.* 2002), using the Bayesian method with nonuniform prior distribution of Zhivotovsky (1999). The significance of the overall estimate was based on 10 000 random permutations of individuals among sites. Finally, we computed D following equation 11 in Jost (2008) and using the estimates of heterozygosity provided in the output of AFLPsurv (using H_t as H_T, and H_w as H_s). D is a measure of differentiation estimated as the proportion of the total diversity that is contained in the average locality, and is independent of within-locality heterozygosity.

Phenotypic characterization of flammability

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

FlamPC1 explained 58.0% of the variance in flammability and was mostly a gradient of time to ignition and mass loss rate (negative) and heat release (positive). FlamPC2 explained 21.5% of the variance and was

highly associated with bulk density (Table S5, Fig. S1, Supporting Information). That is, flamPC1 was more related to small-scale (i.e. twig) flammability, while flamPC2 reflected flammability associated with whole-plant structure. We thus considered that these two PC axes successfully summarize the phenotypic variability in flammability across individuals.

Genetic and phenotypic association

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

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

To estimate the amount of phenotypic variance in flammability explained by these significant loci, for each flammability axis (flamPC1 and flamPC2) we fitted a multiple linear regression against these loci. Finally, to summarize and graphically display the relationship between individual flammability and genotypic variability, each flammability variable was related to a principal coordinate analysis (PCoA) performed with the corresponding set of significant loci. This analysis also allowed us to include site as a random factor and to consider possible spatial autocorrelation. For this, we constructed a matrix of pairwise linear genetic distances between individuals using these loci and performed a PCoA, based on the covariance matrix, in GENALEX. We used the two main orthogonal axes of variation (lociPCo1 and lociPCo2) to represent the genetic distance between individuals. We then fitted, for each flammability indicator (flamPC1 and flamPC2), a mixed effect model to individual plant data, using flammability as the response variable, the genetic distance (lociPCo1 and lociPCo2) as independent variables and site as a random factor; significance was tested using a likelihood ratio test (LRT). Mixed models were fitted with the *nmle* package in R (Pinheiro *et al.* 2013). To evaluate the possible effect of the geographical distance in this regression, we compute the spatial autocorrelation of the residuals (Moran's I statistic) using the *spdep* and *pgirmess* packages of R (Giraudoux 2013); low autocorrelation of the residuals would imply that the regressions are not affected by spatial effects (Diniz-Filho *et al.* 2003). Differences in the axes values between fire regimes for both flammability and genetic axes were also evaluated using a mixed model with site as a random factor and tested with a LRT.

F_{ST} outlier loci method

Our estimation of putative adaptive loci was based on their relationship with flammability. To further support that there is a genetic basis to the phenotypic differences observed in the field, we additionally searched for outlier loci using a population genomic approach (i.e. a phenotype-independent method) as implemented in the software BAYESCAN 2.1 (Foll & Gaggiotti 2008). This method is based on differentiation between populations, highlighting loci with exceptional genetic

differentiation when compared to the neutral expectation. A global analysis based on the AFLP markers was run for the two fire regime categories (a two-deme model: HiFi and NoFi). We pooled sites within the same fire regime because our purpose was to detect allelic variation specifically related to fire. BayeScan automatically tuned model parameters using short pilot runs (20 pilot runs, length 5000). The default chain parameters worked well for our data, so the sample size was 5000 with a thinning interval of 10. We set the prior odds to the neutral model to 1 and set the uncertainty for the inbreeding coefficient (Fis) prior to vary uniformly between 0.3 and 0.7. This is because our study plant has a mixed mating system, as it is self-compatible but pollinated by effective pollinators (large bees) which are highly likely to perform cross-pollination.

Results

A total of 376 polymorphic loci were scored from the nine primer combinations for each of 169 individual plants in the four study sites. A final data set excluding loci where the most frequent allele had a frequency ≥99% comprised 329 AFLP loci and yielded within-site genetic diversity values (H_j, PLP and Br) that were similar among sites (Table 2). Estimates of among-site differentiation showed low genetic differences in spite of geographical distances. The vast majority of the molecular variance occurred within sites (92%), leaving the remaining 8% to variance among sites (AMOVA, $P < 0.001$, overall $\Phi_{PT} = 0.078$). This is confirmed by the other two estimates, overall $F_{ST} = 0.05$ and $D = 0.02$. The site located furthest from the other three, Ares del Maestrat (Table 1 and Table S2, Supporting Information), was also the most genetically distant, as expected. However, paired F_{ST} and Φ_{PT} values between 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 S7, Supporting Information). The remaining sites had paired F_{ST} values between 0.02 and 0.04 and paired Φ_{PT} values between 0.04 and 0.06. In addition, grouping sites with the same fire regime for the analysis (HiFi and NoFi sites) and comparing their F_{ST} estimates also yielded low differentiation ($F_{ST} = 0.02$). These results suggest that geographical separation among our study sites does not prevent gene flow among them. Spatial effects do occur, possibly due to isolation by distance, but our results imply that all sites are interconnected, as expected by the high abundance and the widespread distribution of this species in the study region. Therefore, we pooled all individuals from the four sites for the remaining analyses of genetic and phenotypic association.

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

Table 2 Genetic diversity estimates for each site based on 329 AFLP loci

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

N, number of individuals sampled (genotyped and phenotyped) individuals; PLP, proportion of polymorphic loci; H_j, unbiased Nei's gene diversity (±SE); Br, band richness.

Table 3 Results of the independent logistic regressions (GLM) across individuals of AFLP loci presence/absence against two condensed flammability variables (flamPC1 and flamPC2). Only the results for the loci with statistically significant relationships after correction for false discovery rates are shown (16 for flamPC1 and 13 for flamPC2). See Tables S8 and S9, Supporting Information for the logistic regression obtained with a Bayesian approach. AFLP locus names refer to the primer combination (Table S3, Supporting Information) and the size of the fragment (in bp)

flamPC1				flamPC2			
AFLP locus	Coef.	SE	*P*	AFLP locus	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				

Coef., linear regression coefficient; SE, standard error; *P*, *P*-value.
*AFLP loci are outlier loci detected by the phenotype-independent method in BayeScan.

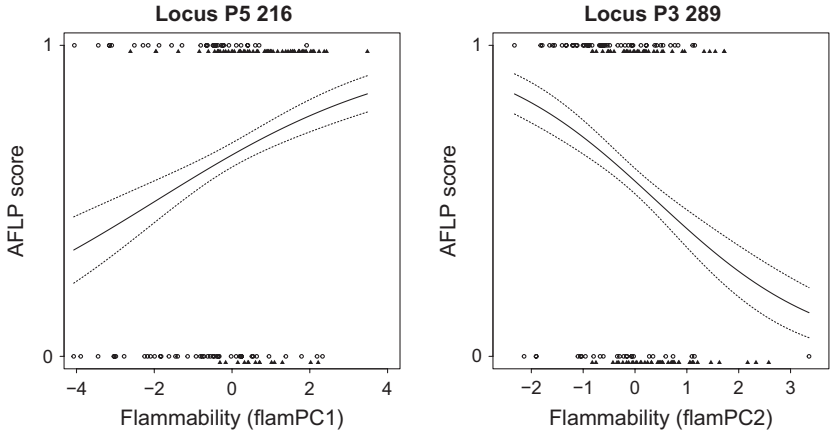

Fig. 1 Relationship between plant flammability (flamPC1, flamPC2) and AFLP scores (1: AFLP locus presence; 0: AFLP locus absence) for loci P5-216 (left) and P3-289 (right) across *Ulex parviflorus* individuals (HiFi in closed triangles, NoFi in open circles). Closed symbols are slightly moved down for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

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

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

Table 4 Summary of the likelihood ratio test (LRT) for the linear mixed-effects model of genotype (lociPCo1 and lociPCo2) against the observed phenotypic variability in flammability (flamPC1 and flamPC2) in *Ulex parviflorus*

	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

AIC, Akaike information criterion.

The phenotypic-independent outlier detection analysis showed that four AFLP loci of the 220 analysed (Table S4, Supporting Information) were outlier loci. All of these four loci were already pointed out in the previous analysis as being related to one or both flammability variables (Table 3). Figure 3 shows an example of one such relationship, where the allelic frequencies of locus P5-216 in the four study sites are strongly correlated with flammability (see also Fig. S2, Supporting Information for a plot with the relationship between this marker and geographical distance between sites). We confirmed that none of the four loci were simply associated with a single site by performing two independent runs in BayeScan, but limited to within HiFi and NoFi sites, respectively (i.e. two sites in each); none of the four loci detected in the overall analysis appeared in these two independent runs (results not shown).

Discussion

Recurrent fires exert a strong evolutionary pressure in postfire obligate seeders because fire kills established individual plants and population persistence is attained by a profuse recruitment from soil or canopy seedbanks (Keeley *et al.* 2011; Moreira & Pausas 2012). In these species, being flammable provides fitness benefits because fires break seed dormancy and open up microsites for recruitment (Bond & Midgley 1995; Schwilk 2003; Moreira *et al.* 2010; Pausas & Moreira 2012; Pausas *et al.* 2012). Here, we showed that, for the postfire obligate seeder *Ulex parviflorus*, an important part of the pheno-

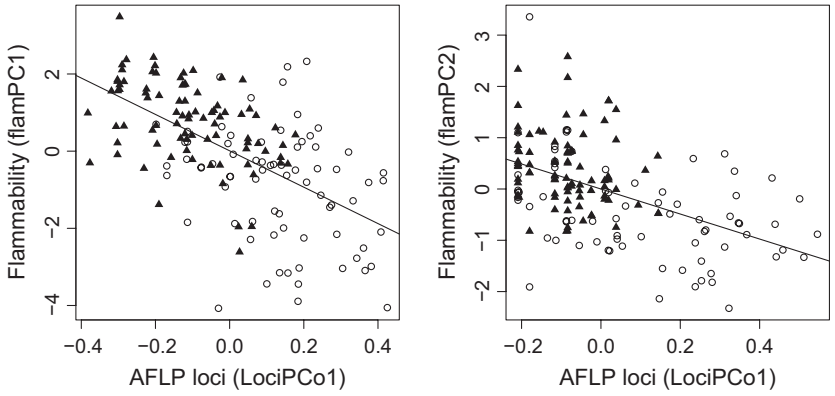

Fig. 2 Relationship between individual *Ulex parviflorus* flammability and genotypic distance based on potentially adaptive AFLP loci (lociPCo1 score), for flamPC1 (left) and flamPC2 (right). The significant ($P < 0.001$) regression lines are also plotted. The two flammability axes (flamPC1 and flamPC2)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

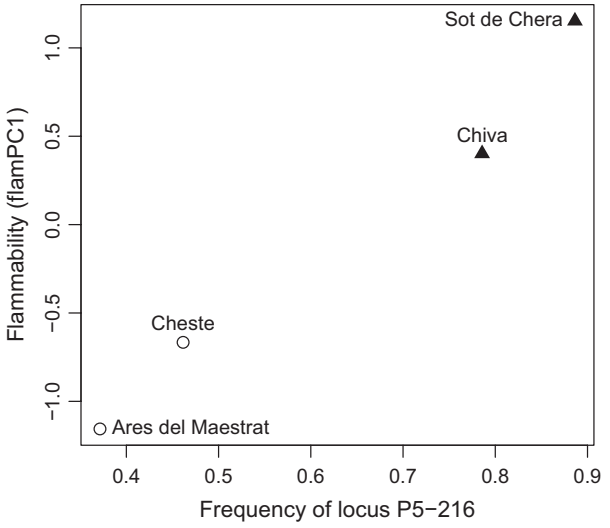

Fig. 3 Relationship between allelic frequency of locus P5-216 and average flammability (flamPC1 score) for the four study sites. Sites are Ares del Maestrat and Cheste (NoFi; in open circles), Sot de Chera and Chiva (HiFi, in closed triangles).

typic variability in plant flammability has a genetic component (at least 42%) and that the genetic variability in putative adaptive loci is structured by differences in fire recurrence. These results, together with the concurrent phenotypic divergence in this species, associated with different fire regimes (Pausas *et al.* 2012), further support the adaptive nature of flammability.

The phenotypic-oriented approach for finding adaptive molecular variation, combining a whole-genome scan analysis with the use of individual phenotypic data, allowed us to suggest that fire might drive changes in allelic frequencies across natural sites and that such variability is associated with particular flammability phenotypes. It is possible that some of the genetic variation could be associated with traits that covary with flammability in response to other factors (e.g. environmental characteristics, geographical distance). However, this is improbable because fire regimes do not vary in parallel with either environmental conditions or with the geographical distribution of the sites (Table 1). Although we used a relatively large number of individuals, one caveat of this study is that it is based on a limited number of sites (two HiFi and two NoFi sites). We limited the analysis to the sites that we were confident of fire history and that individual-level phenotypic variation has been carefully studied.

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

The divergence in flammability in *U. parviflorus*, associated with differences in fire recurrence, occurs in spite

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

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

Evidence of adaptive divergence driven by fire is currently growing (Pausas & Schwilk 2012), although most studies focus on a single trait, serotiny. Pine serotiny varies with fire regime (Hernández-Serrano *et al.* 2013), and there is evidence that variability in common garden trials follows closely that of parental stands (Kuser & Ledig 1987; Ledig *et al.* 2013). In addition, recent genomic scans have detected a strong genetic signal for this trait (Parchman *et al.* 2012; Budde *et al.* 2014). The only other evidence of fire-driven adaptive divergence comes from an annual plant whose seed traits are heritable and vary predictably under different fire regimes (Gómez-González *et al.* 2011). Thus, our results provide the first field evidence supporting that traits enhancing plant flammability may be responding to natural selection driven by fire. This conclusion agrees with recent studies on the crucial role of flammability in key moments of the evolutionary history of plants (Bond & Scott 2010) and specifically in the diversification of plant lineages subject to strong fire pressures (Schwilk & Ackerly 2001; He *et al.* 2011, 2012). Altogether, these various studies point towards the key role of flammability in plant evolution, a characteristic that has often been neglected in the evolutionary literature.

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J.G.P. designed the study; B.M. performed the sampling; B.M. and M.C.C. performed the molecular analyses; B.M., M.C.C. and J.G.P. analysed the data and wrote the first version of the manuscript; M.C.C. and J.G.P. wrote the final version.

Data accessibility

AFLP and phenotypic data: DRYAD entry doi:10.5061/dryad.c62kd.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Flammability variables (time to ignition, mass loss rate, heat released and bulk density) summarized into two orthogonal axes of variation using a principal components analysis (PCA).

Fig. S2 Relationship between allelic frequency of locus P5-216 and average flammability (flamPC1 score, left panel, as in Fig. 3 of the main text) and geographical distance (distance of each site to Ares del Maestrat; right panel).

Table S1 Geographical coordinates (Lat: latitude, Long.: longitude) and summary of the distances (in metres) between the individuals sampled for each site.

Table S2 Pairwise geographical distances between sites (in km).

Table S3 Code, primer combination, number of markers, range sizes (bp) and scoring error rates for each of the nine combinations used.

Table S4 Number of individuals and AFLP loci included in each analysis.

Table S5 Loadings and importance of components for flammability.

Table S6 Pairwise F_{ST} between populations, estimated from 329 AFLP loci (loci present in ≥1% or ≤99% of individuals).

Table S7 Pairwise ΦPT between populations.

Table S8 Allelic frequency for each site and results of the independent logistic regressions across individuals of AFLP loci presence/absence against flammability (flamPC1).

Table S9 Allelic frequency for each site and results of the independent logistic regressions across individuals of AFLP loci presence/absence against flammability (flamPC2).

Protocol S1 AFLP protocol.