Increased fire frequency promotes stronger spatial genetic structure and natural selection at regional and local scales in *Pinus halepensis* Mill

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• Background and Aims The recurrence of wildfires is predicted to increase due to global climate change, resulting in severe impacts on biodiversity and ecosystem functioning. Recurrent fires can drive plant adaptation and reduce genetic diversity; however, the underlying population genetic processes have not been studied in detail. In this study, the neutral and adaptive evolutionary effects of contrasting fire regimes were examined in the keystone tree species *Pinus halepensis* Mill. (Aleppo pine), a fire-adapted conifer. The genetic diversity, demographic history and spatial genetic structure were assessed at local (within-population) and regional scales for populations exposed to different crown fire frequencies.
• Methods Eight natural *P. halepensis* stands were sampled in the east of the Iberian Peninsula, five of them in a region exposed to frequent crown fires (HiFi) and three of them in an adjacent region with a low frequency of crown fires (LoFi). Samples were genotyped at nine neutral simple sequence repeats (SSRs) and at 251 single nucleotide polymorphisms (SNPs) from coding regions, some of them potentially important for fire adaptation.
• Key Results Fire regime had no effects on genetic diversity or demographic history. Three high-differentiation outlier SNPs were identified between HiFi and LoFi stands, suggesting fire-related selection at the regional scale. At the local scale, fine-scale spatial genetic structure (SGS) was overall weak as expected for a wind-pollinated and wind-dispersed tree species. HiFi stands displayed a stronger SGS than LoFi stands at SNPs, which probably reflected the simultaneous post-fire recruitment of co-dispersed related seeds. SNPs with exceptionally strong SGS, a proxy for microenvironmental selection, were only reliably identified under the HiFi regime.
• Conclusions An increasing fire frequency as predicted due to global change can promote increased SGS with stronger family structures and alter natural selection in *P. halepensis* and in plants with similar life history traits.

Key words: Genetic diversity, spatial genetic structure, demographic history, natural selection, fire ecology.

INTRODUCTION

Wildfires influence plant species composition in natural environments and play an important role in the distribution of biomes worldwide (Pausas and Keeley, 2009). In Mediterranean ecosystems, wildfires are a common feature (Keeley *et al.*, 2012); however, the predicted rise in temperatures and reduction in precipitation due to climate change (De Castro *et al.*, 2005) are expected to increase fire recurrence in the coming decades (e.g. Pausas, 2004). Anthropogenic land use change increases fuel availability, and might therefore lead to a shift from fuel-limited to drought-driven fire regimes (Pausas and Fernández-Muñoz, 2012). Hence, studies aiming to understand the effects of fire events on biodiversity are especially important in the face of global change.

Wildfires are major selective drivers shaping adaptive plant traits (Keeley *et al.*, 2011; Pausas, 2015). Adaptive strategies to fire regimes vary substantially in different plant groups and geographic regions (Pausas *et al.*, 2004*a*; Keeley *et al.*, 2011). In Mediterranean-type vegetation, three types of adaptive strategies to fire regime can be distinguished: (1) individuals are affected by fire but they persist through resprouting (resprouters); (2) most individuals are killed by fire and populations mainly persist via a fire-resistant seed bank (seeders); and (3) individuals are protected from the effects of fire through adapted vegetative structures, such as a thickened bark (fire avoiders) (reviewed in Pausas, 2015). Fire response can vary between populations of a species. For instance, some Mediterranean pine species display variable levels of seeding, with some

populations developing canopy seed banks of closed 'serotinous' cones that persist in the canopy for years until high temperatures, often associated with fire, trigger seed release (Lamont *et al.*, 1991; Tapias *et al.*, 2004). Moreover, some pine species combine variable degrees of serotiny with other characters typical of fire avoiders, e.g. a thickened bark, large and protected buds or self-pruning of the lower branches which prevents understorey fires from reaching the canopy easily (Tapias *et al.*, 2004; Hernández-Serrano *et al.*, 2013).

Fire regimes can affect a species' genetic constitution over time through interacting with its life history traits, and adaptive traits in particular. First, frequent fires can produce population bottlenecks by killing individuals before the canopy seed bank recovers, thereby reducing the effective population size and genetic diversity (Whelan, 1995). Secondly, the fire regime can act as a selective driver on adaptive response traits of populations, leaving a signal on specific genes underpinning these traits (Parchman *et al.*, 2012). Thirdly, fire-mediated selection can alter seed traits or dispersal features (Saracino *et al.*, 1997), which will affect the distribution of related individuals or gene copies in a population, i.e. its spatial genetic structure (SGS). Fourthly, the local site conditions, such as shrub cover, aspect, slope, soil depth, ectomycorrhizal distribution and the amount of dead branches on the ground, can influence seedling establishment and survival after fire (Pausas *et al.*, 2004b; Buscardo *et al.*, 2011). This can result in patchy recruitment and heterogeneous selection, which should affect SGS (Epperson, 1995; Linhart and Grant, 1996) and could leave distinct signatures at neutral and relevant adaptive genetic markers (e.g. see van Heerwaarden *et al.*, 2010 for an example of SGS due to microenvironmental selection).

Empirical population genetic studies indicate that in seeder species, soil or canopy seed banks generally preserve population genetic diversity and buffer against demographic fluctuations (Templeton and Levin, 1979; Barrett *et al.*, 2005; Ayre *et al.*, 2009) unless fires are too frequent for the seed bank to recover between fire events (Bradstock *et al.*, 1996). For instance, *Pinus halepensis*, a typical seeder, featured similar levels of genetic diversity in unburnt stands and in stands regenerated after a fire (Schiller *et al.*, 1997). Several studies showed a weak spatial genetic structure in populations regenerated from seed after fire, suggesting a relevant overlap of seed shadows [e.g. for seedling cohorts in *Persoonia mollis* (Ayre *et al.*, 2009) and *Pinus halepensis* (Gershberg *et al.*, 2016); for mature stands of *Nothofagus dombeyi* (Premoli and Kitzberger 2005)], although remnant seed-releasing adults surviving the fire can influence the pattern (Premoli and Kitzberger, 2005; Fajardo *et al.*, 2016). These empirical studies examined the population genetic effects of a single or a couple of fire events, but did not specifically address the evolutionary response of populations to frequent fires. To understand the evolutionary significance of frequent fires on genetic diversity and its spatial structure, it is pertinent to compare populations growing under different fire regimes. We address this gap and, in addition, we compare neutral and functional genetic markers, to explore possible selective effects of fire in a population genetics framework.

In this study, we investigated the impact of fire regime on genetic diversity, demography and genetic structure, including signals of selection, in natural stands of *Pinus halepensis* Mill. (Aleppo pine). The species has been exposed to a long fire history in the Mediterranean basin, which has shaped a mosaic of populations adapted to specific local fire regimes (Tapias *et al.*, 2004; Hernández-Serrano *et al.*, 2013). *Pinus halepensis* generally exhibits a seeder strategy where adults are killed by fire and viable seeds are preserved in serotinous cones in canopy seed banks (Lamont *et al.*, 1991; Tapias *et al.*, 2004). The proportion of serotinous cones and other fire response traits depends on the stands' specific fire regime (Hernández-Serrano *et al.*, 2013). Recruitment is generally massive and simultaneous in the first rainy season after a fire event and depends primarily on the seeds dispersed from the canopy seed bank. In areas where fires are absent or rare, recruitment occurs mainly in gaps based on seeds from non-serotinous cones. The germinability of recently dispersed seeds is high both in undisturbed and in post-fire stands, but it decreases rapidly and is negligible after two rainy seasons, so that *P. halepensis* has only a transient soil seed bank (Daskalakou and Thanos, 1996). We sampled stands exposed to high or low frequency of crown fires (HiFi vs. LoFi) in the eastern Iberian Peninsula and genotyped neutral microsatellites (simple sequence repeats, SSRs) and functional, potentially adaptive single nucleotide polymorphisms (SNPs) defined in coding regions to test the following predictions: (1) HiFi stands should show lower genetic diversity and stronger signs of demographic bottlenecks than LoFi stands if frequent fires hampered the build-up of seed banks. (2) SNPs under fire-mediated selection should display increased differentiation between HiFi and LoFi stands because phenotypic differences in fire-adaptive traits between these stand types (Hernández-Serrano *et al.*, 2013) suggest that differential selection pressures exist. These selection pressures should specifically and differentially affect the allele frequencies of relevant SNPs in HiFi vs. LoFi stands, causing increased differentiation, while neutral markers should only be affected by demographic processes. (3) HiFi stands should show more pronounced SGS than LoFi stands. This prediction arises from the observation of increased spatial aggregation of serotiny in HiFi stands (the same stands as this study; Hernández-Serrano *et al.*, 2013). If this aggregation of serotiny was due to the recruitment of closely related seeds from clumped post-fire dispersal as suggested by Hernández-Serrano *et al.* (2013), then a stronger SGS in HiFi vs. LoFi stands should be expected. (4) SNPs under fire-mediated microenvironmental selection should display a stronger SGS than neutral markers, reflecting clumped recruitment of adapted individuals.

MATERIALS AND METHODS

Study species

Pinus halepensis Mill. (Aleppo pine) is a monoecious conifer tree species native to the Mediterranean basin, where it preferentially occupies dry sites (Tapias *et al.*, 2004). It is a pioneer species, featuring extensive wind-mediated pollen and seed dispersal (Steinitz *et al.*, 2011), and has a high colonization capacity (Goubitz *et al.*, 2004). In regions with frequent stand-replacing crown fires, *P. halepensis* populations show traits typical of the seeder strategy, including an early age of first flowering (4–8 years), retention of dead branches on the stem (which increases the likelihood of crown fires), and high levels of serotiny, i.e. a well-developed canopy seed bank which

ensures post-fire regeneration (Ne'eman *et al.*, 2004; Tapias *et al.*, 2004). In fire-free periods, serotinous cones persist on the trees and can maintain seed viability for >20 years (Tapias *et al.*, 2004). In regions with lower frequency of crown fires, seeder traits are less prevalent, recruitment is not dependent on serotinous cones and occurs mostly in gaps (Tapias *et al.*, 2004; Hernàndez-Serrano *et al.*, 2013).

Study sites and sampling

We chose a regional scale for this study to examine the population genetic effects of fire regimes while controlling for broad-scale processes such as glacial history. The study region in the east of the Iberian Peninsula harbours a single *P. halepensis* gene pool [see Fig. 1; Supplementary Data S1) and comprises populations in close proximity that are affected by contrasting fire regimes (Pausas, 2004; Hernàndez-Serrano *et al.*, 2013). Needles of 371 geo-referenced adult individuals (the same trees as in Hernàndez-Serrano *et al.*, 2013) were collected in 2009 and 2010 in five stands in the eastern, coastal sub-region (<800 m a.s.l.) where the climate is warm and dry and crown fires are common (labelled HiFi hereafter, i.e. high frequency of crown fires), and in three stands in the western, inland sub-region in a sub-humid climate where crown fires are rare (labelled LoFi hereafter, i.e. low frequency of crown fires, Table 1; Fig. 1). Three stands [Sinarcas (LoFi), Serra Calderona (HiFi) and Eslida (HiFi)] were intensely sampled with 64–67 samples/stand, hereafter referred to as 'Intensive Study Plots'

(ISPs), while 31–39 samples/stand were collected in the other stands (Table 1). Recent reports document that >50 % of the study region at < 800 m a.s.l. (HiFi conditions) burned at least once during the 1978–2001 period, while the proportion was only about 15 % for >800 m a.s.l. (LoFi conditions; Pausas, 2004; Pausas and Fernàndez-Muñoz, 2012). All stands had similar population densities, of 285–441 trees ha^{-1}. Needles were dried with silica gel and DNA was extracted using the Invisorb DNA Plant HTS 96 Kit (Stratec, Birkenfeld, Germany).

Genetic markers

Nuclear microsatellites. Samples were genotyped at nine SSRs (Supplementary Data S2). Forward primers were 5^0-end-labelled with fluorochromes and amplification was performed using the Qiagen Multiplex PCR Kit (Qiagen, Venlo, The Netherlands) or Promega GoTaq mix (Promega, Madison, WI, USA) (S2). Amplified fragments were separated using an ABI 3730 genetic analyser (Applied Biosystems, Carlsbad, CA USA) and fragment sizes were determined with reference to the GeneScan™–500 LIZ$^{\Psi}$ Size Standard (Applied Biosystems) using GeneMapper software version 4.0 (Applied Biosystems).

Single nucleotide polymorphisms. For SNP genotyping, we genotyped a 384-plex SNP assay designed from the aligned transcriptomic sequences of two individuals with extreme phenotypes regarding fire adaptation (specifically, with high
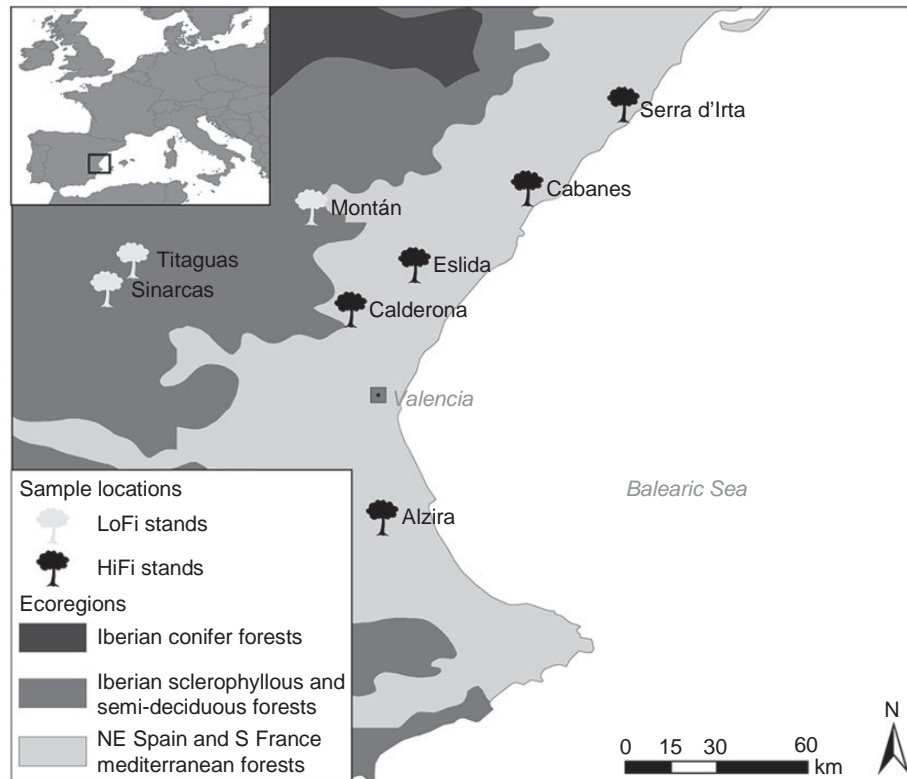

F_IG_. 1. Study stands of *Pinus halepensis* in the Eastern Iberian Peninsula belong to different ecoregions. Coastal stands experience a high frequency of crown fires (HiFi) while inland stands experience a low frequency (LoFi).

TABLE 1. *Genetic diversity of natural* Pinus halepensis *stands*

Stand	Fire regime	Latitude	Longitude	n	SSRs			SNPs	
					H_E (s.e.)	R_S (s.e.)	F_{IS}	H_E (s.e.)	F_{IS}
Alzira	HiFi	39·12415	−0·38785	39	0·383 (0·042)	3·467 (0·590)	−0·013 n·s·	0·290 (0·011)	0·027*
Cabanes	HiFi	40·10132	0·04182	31	0·341 (0·049)	2·912 (0·368)	−0·046 n·s·	0·244 (0·012)	0·083***
Serra Calderona	HiFi	39·74162	−0·48395	64	0·470 (0·042)	3·681 (0·803)	0·044 n·s·	0·300 (0·001)	0·071***
Eslida	HiFi	39·87070	−0·29247	67	0·359 (0·051)	2·606 (0·410)	−0·006 n·s·	0·266 (0·001)	0·054***
Serra d'Irta	HiFi	40·35106	0·32312	39	0·461 (0·045)	3·854 (0·871)	−0·002 n·s·	0·280 (0·011)	0·036*
Montàn	LoFi	39·79760	−1·20414	31	0·395 (0·064)	3·230 (0·410)	0·043 n·s·	0·280 (0·011)	0·065***
Sinarcas	LoFi	40·04873	−0·59526	67	0·441 (0·055)	3·342 (0·689)	−0·073 n·s·	0·289 (0·001)	0·023*
Titaguas	LoFi	39·88786	−1·12823	33	0·431 (0·063)	3·857 (0·924)	0·107*	0·280 (0·011)	0·059***

n, sample size; H_E, gene diversity and its standard error (s.e.); R_S, allelic richness standardized to a sample of 22 diploids; F_{IS}, inbreeding coefficient (significance test for F_{IS} observed > F_{IS} expected: *$P < 0.05$; ***$P < 0.001$; n.s., not significant).

and low numbers of serotinous cones; Pinosio *et al.*, 2014). The 384-plex also included 144 SNPs from candidate genes obtained by resequencing. SNP typing was conducted for all samples with IlluminaVeraCode™ technology. The conversion rate for the SNP assay was relatively high (approx. 66 %), and high-quality genotypes were obtained for 251 polymorphic SNPs (GC_{50} score of approx. 82 %) representing 219 genes (including 88 candidate genes, with one or two SNPs assayed per gene).

Genetic diversity

The allelic richness (R_S), i.e. the sample size-adjusted mean number of alleles per locus, was computed for SSRs using FSTAT 2.9.3.2 (Goudet, 1995). For both SSRs and SNPs, genetic diversity (H_E) was calculated in each stand using GENEPOP 4.0 (Raymond and Rousset, 1995). The fixation index (F_{IS}) was computed, and deviation from zero (Hardy–Weinberg genotypic proportions) was tested using 10 000 permutations of alleles within populations in SPAGeDi 1.4c (Hardy and Vekemans, 2002). Differences for H_E, R_S and F_{IS} between groups of HiFi and LoFi stands were assessed using permutation tests in FSTAT.

Demographic history

Simple sequence repeats were used to assess demographic history in each stand using complementary approaches. First, Cornuet and Luikart's (1996) T_2 statistic, reflecting the deviation of gene diversity from expectations at demographic equilibrium, was computed using the Two-Phase Model (TPM) with default parameter settings in Bottleneck 1.2.02 (Piry *et al.*, 1999) and significance was tested using Wilcoxon's signed rank test. Secondly, Garza and Williamson's (2001) M was calculated as the ratio of the number of alleles over the allele size range using ARLEQUIN 3.5.1.3 (Excoffier and Lischer, 2010). After a severe bottleneck event, M is predicted to decline because the number of alleles should decrease faster than the allele size range. A value below the critical $M_C \frac{1}{4} 0.68$ indicates a bottleneck event (Garza and Williamson, 2001). While T_2 is most appropriate to detect recent, weak bottlenecks, M is best suited to detect older and longer-lasting bottlenecks (Williamson-Natesan, 2006). Finally, a coalescent framework

and Approximate Bayesian Computation (ABC) were used to examine historical population size changes in the ISPs and visualize them in skyline plots (Ho and Shapiro, 2011; see details in Supplementary Data S3).

Testing for regional-scale selection

To assess whether any SNPs (taking into account 179 SNPs with a minimum allele frequency >0·1) were under fire-mediated selection at the regional scale, we used an outlier detection approach based on F-statistics in a hierarchical island model (Excoffier *et al.*, 2009) as implemented in ARLEQUIN 3.5 (Excoffier and Lischer, 2010), grouping HiFi vs. LoFi populations. Outlier loci with unusually strong differentiation were detected by comparison with a 99 % null distribution envelope for F_{ST}. In addition, we used the Bayesian approach implemented in BAYESCAN 2.1 (Foll and Gaggiotti, 2008) to identify high differentiation outliers. A false discovery rate (FDR) approach based on posterior odds ratios (analogous to Bayes factors) was employed for outlier identification.

Fine-scale spatial genetic structure

Spatial genetic structure patterns. Fine-scale SGS was assessed in all stands separately for SSRs and SNPs, as described by Vekemans and Hardy (2004). Kinship coefficients F (Loiselle *et al.*, 1995) were calculated in SPAGeDi 1.4c for all pairs of individuals and regressed on the logarithm of spatial distance. Significance of SGS was tested by comparing the regression slope b with its distribution obtained from 10 000 permutations of individual locations. The strength of SGS was estimated as $Sp \frac{1}{4} b/(1 − F_1)$ (Vekemans and Hardy, 2004) where F_1 is the kinship coefficient in the first distance class. To test the hypothesis of a stronger SGS in HiFi populations, expected if dispersal and recruitment patterns were clumped due to high fire frequency as suggested by Hernàndez-Serrano *et al.* (2013), we conducted SGS analysis using SPAGeDi within each stand type by grouping pairwise comparisons across stands within fire regimes. Differences in SGS between fire regimes were tested with t-tests on per-locus b and comparison of 95 % jackknife confidence intervals (CIs) of b (a steeper slope indicating stronger SGS; Vekemans and Hardy, 2004). These analyses

increased the number of pairwise comparisons in each analysis and thus resulted in higher power.

Testing for microenvironmental selection. Selective processes that operate spatially during recruitment may specifically affect (some) functional SNP markers and thereby the SGS pattern they display. If spatially explicit microenvironmental selection occurred and left a detectable signal, the adaptively relevant SNPs should show significantly stronger SGS than neutral markers (see van Heerwaarden *et al.*, 2010). We first compared global SGS between SNPs and SSRs by comparing the jack-knife 95 % CIs of *b* (as above). These CIs take marker power implicitly into account as they depend on the number and power of markers (see SPaGeDi User Manual). Secondly, as each locus represents an independent estimate of SGS, we applied Welch's *t*-test for (unequal) samples with unequal variances (Welch, 1947) on per-locus *b* values obtained from SNPs vs. SSRs. To investigate further whether any particular SNPs were candidates for spatially explicit microenvironmental selection, we identified the loci that displayed exceptionally strong SGS within stands, namely those with *b* smaller than [first quartile (*b*) – 1·5×interquartile range], as inferred with the 'box-plot' function in R, and compared them across stands. Finally, we applied an FDR approach to the *P*-values of *b* using the package 'fdrtool' (Strimmer, 2008) and compared the retained SNPs (i.e. those with a significant *q*-value) across stands.

Annotation of SNP markers

Annotations for candidate SNPs putatively under selection at both regional and local spatial scales were obtained by retrieving their corresponding full-length transcripts and annotation (Pinosio *et al.*, 2014) as well as by BLASTX searches in NCBI's GenBank.

RESULTS

Genetic diversity and demographic history

Fire regime had no effect on allelic richness, genetic diversity or the inbreeding coefficient; these statistics were similar across all *P. halepensis* stands (Table 1), and the permutation tests to assess differences between HiFi and LoFi stands were non-significant. SSR data indicated Hardy–Weinberg genotypic proportions in seven out of the eight stands (see also Supplementary Data Table S2·2) while SNP data revealed a weak but significant homozygosity excess in all stands. A demographic signal related to fire regime was not observed (Table 2). Recent bottleneck signals were detected in all stands under the TPM in the Bottleneck program. The Garza–Williamson *M* ratio, which typically detects old and long-lasting population size declines, did not show strong evidence of bottlenecks. The ABC skyline plots were in agreement with these results; they were visually similar across stands and showed a recent population size decline (Supplementary Data Fig. S3·1).

TABLE 2. *Bottleneck tests for SSR markers in* Pinus halepensis

Stand	Fire regime	T_2	*P*-value	*M* (s.e.)
Alzira	HiFi	1.859	0.002	0.679 (0.280)
Cabanes	HiFi	2.410	0.002	0.670 (0.261)
Serra Calderona	HiFi	3.548	<0.001	0.735 (0.247)
Eslida	HiFi	3.901	<0.001	0.657 (0.259)
Serra d'Irta	HiFi	3.052	<0.001	0.672 (0.349)
Montán	LoFi	2.471	0.002	0.685 (0.243)
Sinarcas	LoFi	3.805	0.002	0.715 (0.256)
Titaguas	LoFi	2.253	0.002	0.681 (0.238)

T_2, bottleneck statistic of Cornuet and Luikart (1996), *P*-value of the Wilcoxon signed rank test (one tail for *H* excess) under the Two-Phase Model (TPM) calculated in Bottleneck software; *M*, ratio of the number of alleles over the allele size range; a bottleneck is indicated for values below the critical M_C ¼ 0·68.

Signs of selection at the regional scale

The hierarchical model contrasting HiFi vs. LoFi stands in ARLEQUIN identified four high F_{ST} outlier SNPs based on a 99 % confidence envelope (Fig. 2). Of these, three had $P < 0·001$ and $F_{ST} > 0·15$, for an overall multilocus F_{ST} of 0·03. The same three loci were retained in BAYESCAN, with $q < 0·05$, and two of them were located on the same transcript, coding for a b-tubulin; the third coded for phytochrome N (Table 3).

Genetic structure and signs of selection at the local scale

Significant SGS was found across HiFi or LoFi stands at both SSRs and SNPs (Table 4; Figs 3 and 4A). Although the SGS was weak in all stands, our hypothesis of increased SGS for the HiFi regime was confirmed with SNPs: HiFi stands displayed approximately three times stronger SGS than LoFi stands, *Sp* ¼ 0·0094 vs. *Sp* ¼ 0·0027 (Table 4; non-overlapping 95 % CIs of regression slope *b*, Fig. 4A; Student's *t*-test: *t* ¼ –6·067, d.f. ¼ 485, *P* ¼ 4·16e-09). For SSRs, which had lower power (see Supplementary Data S4 for a simulation approach comparing power at SNPs and SSRs), SGS did not differ between fire regimes (Table 4; Fig. 4A; Student's *t*-test: *t* ¼ –0·028, d.f. ¼ 6, *P* ¼ 0·489). At the population level, SNPs displayed significant SGS in four HiFi and in one LoFi stand, while SSRs displayed significant SGS in one HiFi and one LoFi stand (Table 4).

The search for signals of microenvironmental selection revealed a numerically stronger SGS at functional SNPs than at anonymous SSRs across HiFi stands; however, the difference was only marginally significant (*Sp* ¼ 0·0094 vs. *Sp* ¼ 0·0045, Table 4; overlapping CIs for *b*, Fig. 4A; Welch's *t*-test: *t* ¼ –1·508, d.f. ¼ 10, *P* ¼ 0·081). At the population level, two HiFi stands, Alzira and Serra Calderona, displayed stronger SGS at SNPs than at SSRs, suggesting spatially explicit microenvironmental selection detectable at the ensemble of the SNP data (Table 4; non-overlapping 95 % CIs for *b*, Fig. 4B; Welch's *t*-test: *t* ¼ –2·7451, d.f. ¼ 11·418, *P* ¼ 0·009 in Serra Calderona and *P* ¼ 0·058 in Alzira). Low precision of SSRs for SGS estimation, as illustrated by their large CIs (see also S4), may have prevented significant tests in other populations, in particular Cabanes (Fig. 4B).

To explore signals of microenvironmental selection at individual SNPs, we examined outliers for within-population SGS
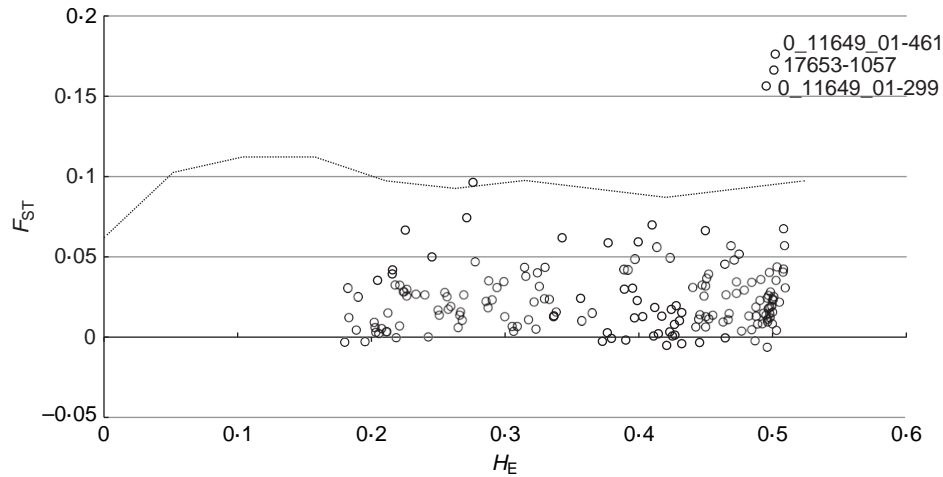
FIG. 2. Plot of F_{ST} as a function of heterozygosity at SNP loci, as determined in a hierarchical island model contrasting HiFi and LoFi stands. Three F_{ST} outliers lie well above the 99 % confidence envelope.

TABLE 3. *Candidate SNP loci for selection at the regional or local scale and their annotation*

SNP name*	Scale of selection	Transcript or amplicon	SNP position (bp)	Best NCBI hit (BLASTx)			Type of change	Accession number[†]
				Locus	E-value	Annotation		
0_11649_01-461	Regional	0_11649_01	461	AIZ74332	5E-75	b-Tubulin	G/A (syn)	2133040722
17653-1057	Regional	17653	1057	AFV79549	0	Phytochrome N	C/T (syn)	2133040723
0_11649_01-299	Regional	0_11649_01	299	AIZ74332	5E-75	b-Tubulin	G/T (intron)	2133040736
54808–2616	Local	54808	2616	AEO97206	0	Endo-1,4-b-glucanase	C/T (syn)	2133040724
0_7183_01-253	Local	0_7183_01	253	ADM74568	4E-15	Phospholipase A2-like partial	C/G (non-syn) Val ! Leu	2133040725
12329-974	Local	12329	974	AJP06241	0	Auxin transporter AUX4	C/G (syn)	2133040726
45843-480	Local	45843	480	AAC32110	9E-92	Hypothetical protein	A/G (syn)	2133040727
32435-5607	Local	32435	5607	XP_006848562	0	ATP-binding protein	C/T (non-syn) Tyr ! Cys	2133040728
15979-616	Local	15979	616	ABS88990	0	Cobra-like	A/T (syn)	2133040729
8968-3592	Local	8968	3592	AAX16014	0	Trehalose-6-phosphate synthase	C/A (5' utr)	2133040730
0_12535_01-285	Local	0_12535_01	285	NA	NA	NA	G/A (NA)	2133040731
11111-2414	Local	11111	2414	NA	NA	NA	A/C (NA)	2133040732
32435-692	Local	32435	692	XP_006848562	0	ATP-binding protein	C/T (syn)	2133040733

NA, unknown/data unavailable; syn, synonymous; non-syn, non-synonymous; 5⁰ utr, 5⁰ untranslated region.   *SNP names correspond to Pinosio *et al.* (2014).   [†] Accession numbers as provided by NCBI's dbSNP (ss).

TABLE 4. *Fine-scale spatial genetic structure (SGS) and serotiny phenotype autocorrelations (from Hernàndez-Serrano et al., 2013) in all LoFi and HiFi stands of* Pinus halepensis

Location	Fire regime	Serotiny		SSRs			SNPs			Welch test, H1: $b_{SNPs} < b_{SSRs}$ P-value
		Slope	P-value	b	Sp	P-value	b	Sp	P-value	
Alzira	HiFi	−0·236	<0·001	0·0072	−0·0070	n.s.	−0·0068	0·0068	0·0012	0·058
Cabanes	HiFi	−0·260	<0·001	0·0061	−0·0062	n.s.	−0·0156	0·0160	0·0003	0·092
Serra Calderona	HiFi	−0·031	n.s.	−0·0011	0·0011	n.s.	−0·0107	0·0112	<0·0001	0·009
Eslida	HiFi	−0·052	0·027	−0·0104	0·0109	0·0027	−0·0100	0·0104	<0·0001	n.s.
Serra d'Irta	HiFi	−0·099	0·021	−0·0049	0·0049	n.s.	−0·0020	0·0020	0·0951	n.s.
All HiFi	HiFi			−0·0045	0·0045	0·0218	−0·0092	0·0094	<0·0001	0·081
Montàn	LoFi	0·025	n.s.	−0·0163	0·0169	0·0377	−0·0137	0·0143	<0·0001	n.s.
Sinarcas	LoFi	−0·020	n.s.	−0·0030	0·0030	n.s.	−0·0005	0·0005	n.s.	n.s.
Titaguas	LoFi	0·026	n.s.	−0·0047	0·0048	n.s.	0·0027	−0·0027	n.s.	n.s.
All LoFi	LoFi			−0·0055	0·0055	0·0322	−0·0028	0·0027	0·0031	n.s.

b, regression slope; Sp, intensity of the SGS; P-values of regression slope b, n.s., not significant $P > 0·1$.
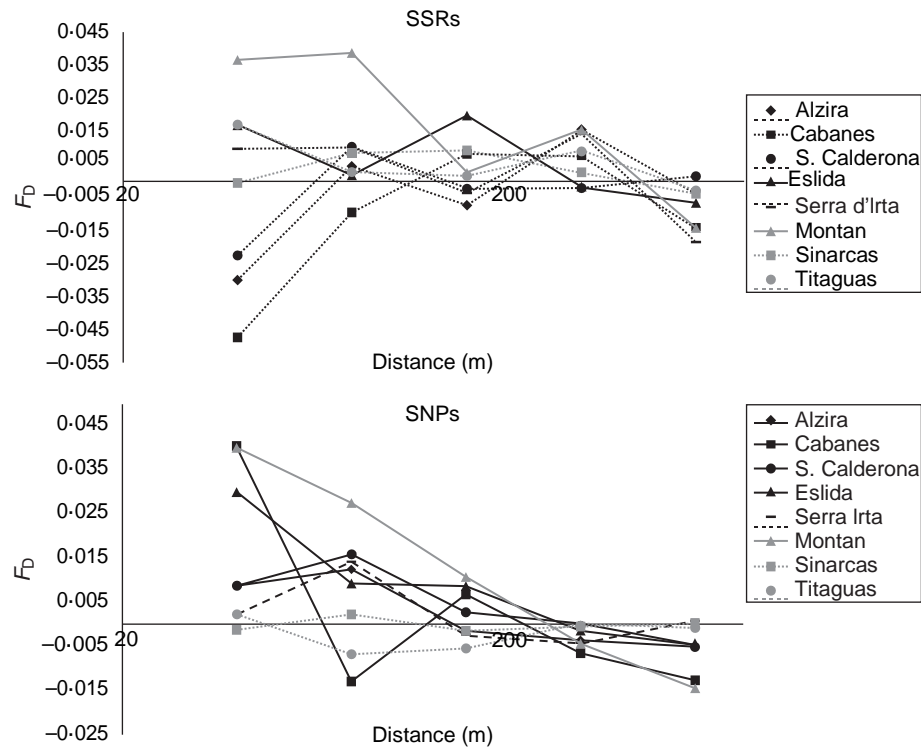
FIG. 3. Spatial genetic structure in *P. halepensis* stands using SSRs and SNPs. The average kinship coefficient F_D was plotted against the logarithm of the geographic distance classes between individuals. The colours of the curve indicate the fire regime, grey for low crown fire frequency (LoFi) and black for high crown fire frequency (HiFi); the symbols indicate the sample location. Significance of the SGS pattern is indicated by continuous lines ($P < 0.05$), marginal significance by a dashed line ($P < 0.1$) and non-significance by dotted lines. S. Calderona, Serra Calderona.

and found 5–20 SNPs with strongly negative *b* in each population (but no outliers for the opposite tail of *b*, Supplementary Data Fig. S4.3). Nine outlier SNPs were shared among at least two populations, and two of them were shared among three populations (Table 5). The more stringent FDR approach revealed that individual HiFi stands had between 0 and 32 SNPs with significant *q*-values (none in Serra d'Irta and in Alzira, one in Cabanes, six in Serra Calderona and 32 in Eslida), while LoFi stands did not feature any SNP with a significant *q*-value. Three of the SNPs with $q < 0.05$ were significant in two populations (Serra Calderona and Eslida), two of them overlapping with the previous set of nine strongly structured SNPs (Table 5).

The annotation of the retained SNPs (Table 3) revealed that two of these candidate SNPs are located on the same transcript (32435) coding for an ATP-binding protein, with SNP 32435-5706 coding for an amino acid change. A further non-synonymous change occurred in a phospholipase A2-like protein coded for by SNP 0_7183_01-253. Several candidate SNPs showed synonymous mutations, such as SNP 15979-616, part of a cobra-like gene, or were located in untranslated regions, e.g. SNP 8968-3592 belonging to a gene coding for trehalose-6-phosphate synthase. No outlier candidate SNP or transcript detected at the local scale overlapped with those identified at the regional scale above.

DISCUSSION

In this work, we showed that different regimes of crown fire frequency did not lead to differences in genetic diversity or inferred demographic history in natural populations of *Pinus halepensis*. However, candidate SNPs for fire-mediated selection were detected at the regional scale. Despite a weak SGS in all *P. halepensis* populations, HiFi stands displayed a stronger spatial genetic structure than LoFi stands, suggesting a more clumped recruitment of related individuals under HiFi. Furthermore, we identified SNPs with exceptionally strong or significant SGS within several populations, indicating that microenvironmental selection possibly contributed to enhancing local genetic structure.

Levels of genetic diversity are maintained under high fire frequency

We expected a reduction in genetic diversity and stronger bottleneck signals in Mediterranean pine stands exposed to frequent crown fires. However, genetic diversity and estimates of effective population sizes were not affected by fire regime in natural *P. halepensis* stands. Similar findings have been reported for the fire-adapted obligate seeder *Ulex parviflorus* (Moreira *et al.*, 2014). Genetic diversity can be preserved through frequent fires because of adaptations to fire, such as an early age of first flowering and a diverse canopy seed bank due to serotiny (Templeton and Levin, 1979; Ayre *et al.*, 2010), and because of high levels of gene flow (Goubitz *et al.*, 2004; Shohami and Nathan, 2014). In the Proteaceae species *Banksia hookeriana*, for example, genetic diversity in a 9-year-old canopy seed bank accumulated rapidly: the first reproduction event
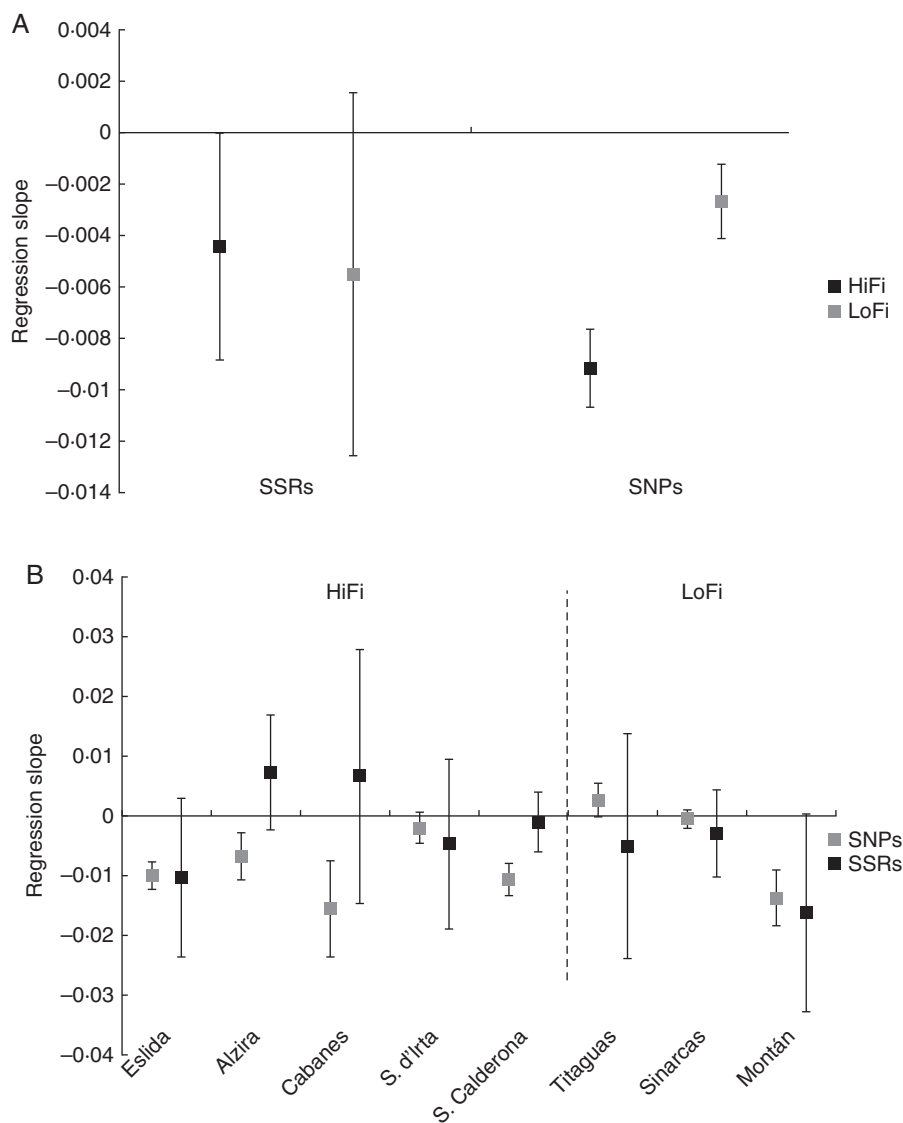
FIG. 4. SGS estimates expressed as mean jackknife regression slopes *b* and their 95 % confidence intervals (CIs); a negative regression slope with CIs not overlapping zero indicates significant SGS. (A) Within-stand SGS across all *Pinus halepensis* HiFi (black symbols) or LoFi (grey symbols) stands. HiFi stands had stronger SGS (more negative *b*) than LoFi stands at SNPs but not at SSRs. (B) Comparison of observed SGS between SSRs and SNPs by population. In the HiFi stands of Alzira and Serra Calderona, non-overlapping CIs between markers suggest that different evolutionary processes operate on SSRs vs. SNPs. S. d'Irta, Serra d'Irta; S. Calderona, Serra Calderona.

after fire already accounted for >80 % of the genetic diversity detected (based on the Shannon index *I*) and genetic variation between seed crop years was small (Barrett *et al*., 2005). In *P. halepensis*, we suggest that abundant pollen gene flow contributes to a fast post-fire recovery of a diverse canopy seed bank, in agreement with higher pollination distances observed in the vegetation-free post-fire landscape (Shohami and Nathan, 2014).

Demographic history

Recent bottleneck signals were detected in all stands independently of fire regimes. The ABC skyline plots suggested that the bottlenecks occurred in the latest tens to hundreds of generations, more recently than the 2000–3000 generations old bottlenecks that characterize *Taxus baccata*, a conifer that does not occur in fire-prone environments (Burgarella *et al*., 2012). Assuming a generation time of 25 years in *P. halepensis* (see Willyard *et al*., 2006), the bottlenecks in our study would correspond to post-glacial or Holocene events (i.e. more recently than the last glacial maximum approx. 20 000 years BP). This dating must be interpreted with caution as it depends on the assumed generation times and mutation rates. The bottleneck timing is potentially congruent with the Late Pleistocene or post-glacial recolonization of the Western Mediterranean basin from the Balkan Peninsula suggested for this species (Bucci *et al*., 1998; Morgante *et al*., 1998). The bottleneck signatures

TABLE 5. *Candidate SNP loci for microenvironmental selection and criteria for their selection*

| | HiFi | | | | | | | | | | LoFi | | | | | | All HiFi | | All LoFi | |
| | Alzira | | Cabanes | | Serra Calderona | | Eslida | | Serra d'Irta | | Montán | | Sinarcas | | Titaguas | | | | | |
	b	P-value	b	P-value	b	P-value	b	P-value	b	P-value	b	P-value	b	P-value	b	P-value	b	P-value	b	P-value
SNPs with outlier b																				
54808-2616							-0·048	**					-0·035	**	-0·062	*	-0·024	**	-0·039	**
12329-974			-0·134	**											-0·064	*				
45843-480					-0·073	**	-0·067	***					-0·034	*			-0·048	***	-0·029	**
32435-5607	-0·063	*			-0·079	***											-0·038	***		
15979-616							-0·067	***					-0·046	**			-0·033	***	-0·026	*
8968-3592	-0·069	*											-0·029	*						
0_12535_01-285			-0·139	**	-0·059	**							-0·025	*						
11111-2414					-0·075	***							-0·025	*			-0·018	*	-0·021	*
32435-692	-0·063	*															-0·037	**	-0·025	*
SNPs with q < 0.05																				
0·7183_01-253					-0·047	**	-0·028	*									-0·029	***		
45843-480					-0·073	**	-0·067	*									-0·048	***		
32435-5607					-0·079	***	-0·024	*									-0·038	***		

The regression slope b is given for SNPs with outlier b (strong SGS, upper part of table) detected in at least two stands or with significant SGS after FDR correction (q < 0.05, lower part of table). SNPs retained according to both criteria are indicated in bold. *P*-values: *P < 0.05; **P < 0.01; ***P < 0.001.

could additionally reflect a population size reduction during a mid-Holocene phase of low fire incidence in the East Iberian study region, during which the fire-dependent *P. halepensis* was near-absent (but it was abundant before and after this period; Carrión and Van Geel, 1999). There is additional evidence that oak forest replaced pine forest in the south-eastern Iberian Peninsula between approx. 8000 and 5500 BP, concomitantly with a reduction in fire frequency (Vannière *et al.*, 2011). It appears thus that the bottlenecks detected in the study stands do not reflect recent fire events but are instead explained in terms of regional population genetic history.

Population genetic effects of frequent fires

We detected two main effects of different crown fire regimes: signals of selection at the regional scale and increased spatial genetic structure within HiFi stands. The SNPs under selection between HiFi and LoFi stands are located in genes involved in wood formation (b-tubulin; Oakley *et al.*, 2007) and circadian rhythms (phytochrome N; Mathews, 2010). Although they do not encode amino acid substitutions, synonymous substitutions and mutations in untranslated regions have been shown to affect phenotypes through various mechanisms (Hunt *et al.*, 2014). Therefore, our candidates could potentially play a role in the expression of fire-related traits, such as increased serotinous cone formation observed under HiFi (Hernández-Serrano *et al.*, 2013).

Higher SGS under frequent crown fires was expected because of previous indications of clumped post-fire recruitment near burnt adults (Ne'eman and Izhaki, 1998) and significant spatial autocorrelation of fire-related phenotypes (i.e. serotiny) in our study stands (Hernández-Serrano *et al.*, 2013; also reported in Table 4). Hernández-Serrano *et al.* (2013) suggested that spatial autocorrelation for serotiny in HiFi stands reflected restricted overlapping of post-fire seed rain shadows. Our results sustain this explanation (but see below), demonstrating a high genetic relatedness at short distances, thus suggesting clumped recruitment of related individuals around their mothers in post-fire conditions where space and resources are, in principle, available. In LoFi stands, in contrast, weak SGS seems to indicate a more efficient genetic mixing in pollination and seed dispersal. Thus, the more gradual, fire-independent recruitment in small gaps in these stands does not result in marked clumped family structures, in contrast to observations of increased genetic relatedness at short distances in old-growth vs. post-fire stands of other light-dependent tree species (Premoli and Kitzberger, 2005; Fajardo *et al.*,2016).

Dispersal-related differences between fire regimes should similarly affect SGS at SNPs and SSRs in the absence of selection. In our study, SSRs did not detect any difference in SGS between fire regimes, which can be largely explained by their lower power (see Fig. 2; S4). We detected numerically stronger SGS at SNPs than at SSRs in HiFi stands (P ¼ 0·081), which raises the possibility that spatially explicit selective processes could be involved in enhancing SNP-based SGS. The assayed SNPs are enriched in genes that are potentially relevant for adaptation to fire regimes, as many SNPs were designed from the transcriptomes of individuals with

extreme serotiny phenotypes (Pinosio *et al.*, 2014). *Pinus halepensis* is a strongly fire-dependent species, with a high risk of post-fire adult mortality and opportunity for microenvironmental selection in relation to the expression of fire-related traits (Fernandes *et al.*, 2008). Heterogeneous, patch-dependent selection can result in increased SGS (Epperson, 1995; Linhart and Grant, 1996), which should particularly show at genes targeted by selection, or at loci linked to them. Selection at short spatial scale can be caused by microenvironmental factors that influence post-fire seedling establishment and survival (Pausas, 2004; Buscardo *et al.*, 2011). In *P. halepensis* in particular, abundant post-fire regeneration was observed in sites with large amounts of branches on the ground (which create appropriate microclimatic conditions), with northern aspects, with high pre-fire basal area and on terraced slopes (Pausas *et al.*, 2004*b*).

When examining specific SNPs for signs of microenvironmental selection, we retained nine loci with either particularly strong or significant SGS in at least two populations, suggesting the occurrence of parallel spatially explicit selection processes in the stands concerned. Some of these SNPs were detected in LoFi stands, suggesting that selection processes responded to other microenvironmental features (e.g. soil depth, water, nutrient and light availability or biotic interactions) besides fire. We indeed observed that regional and local-scale selection targeted different genes. Among the nine retained candidate SNPs at the local scale, two are located on the same transcript, coding for an ATP-binding protein, including a non-synonymous SNP. Mutations in ATP-binding sites have previously been related to stress response (Cross and Templeton, 2004). A second non-synonymous SNP was located in a region coding for a phospholipase A2-like protein. Phospholipases A2 (PLA2s) mediate signalling cascades during plant growth and development, and intervene in biotic and abiotic stress responses, particularly in drought stress (Verlotta *et al.*, 2013). We further detected a synonymous substitution in a cobra-like gene probably involved in cell formation and cellulose content (Thumma *et al.*, 2009) and a mutation in an untranslated region of a trehalose-6-phosphate synthase-coding gene probably involved in abiotic stress responses (Iordachescu and Imai, 2008). These functions could be relevant to cope with different types of biotic and abiotic stresses such as those projected under impending climate change (e.g. drought, fire); therefore, they deserve further attention in the future.

Overall, we detected few SNPs with robust evidence for selection. This result is in agreement with recent work on fire adaptation in pines where models that combine the expected small effects of multiple loci (i.e. polygenic adaptation) predict fire-related traits more successfully than single-locus models (e.g. for serotiny, Parchman *et al.*, 2012; Budde *et al.*, 2014). Future research into microenvironmental selection associated with fire in this system is a promising avenue, and could proceed by examining the combined effects of multiple loci on fire phenotypes within populations.

CONCLUSIONS

Predicting plant responses to future fire regime changes is challenging and complex (Keeley *et al.*, 2012). With global change, fire frequency is expected to increase, including in areas where fire has not had an important impact so far (e.g. Pausas, 2004; Pausas and Keeley, 2014). In a region with a long fire history, *P. halepensis* stands retained their genetic diversity under frequent fires and responded to fire-related and other selection pressures at regional and local scales. Our results suggest that increases in fire frequency in regions that are so far little affected by fire can lead to altered recruitment patterns with stronger family structures and response to natural selection in *P. halepensis* and in other species with similar life history traits.

SUPPLEMENTARY DATA

See final version of the paper

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Supplementary Information

S1 Population genetic structure

A Bayesian clustering algorithm implemented in STRUCTURE 2.2 (Pritchard 2000) was used on the SSR data of all HiFi and Lofi stands, to identify population genetic structure in the study region. We ran an admixture model with correlated allele frequencies between clusters. Ten runs were performed for each number of clusters $K=1$ to $K=5$ with a burn-in length of 100 000 and a run length of 200 000 iterations.

The STRUCTURE analysis indicated a single gene pool in the study region as expected from the post-glacial history of these stands.
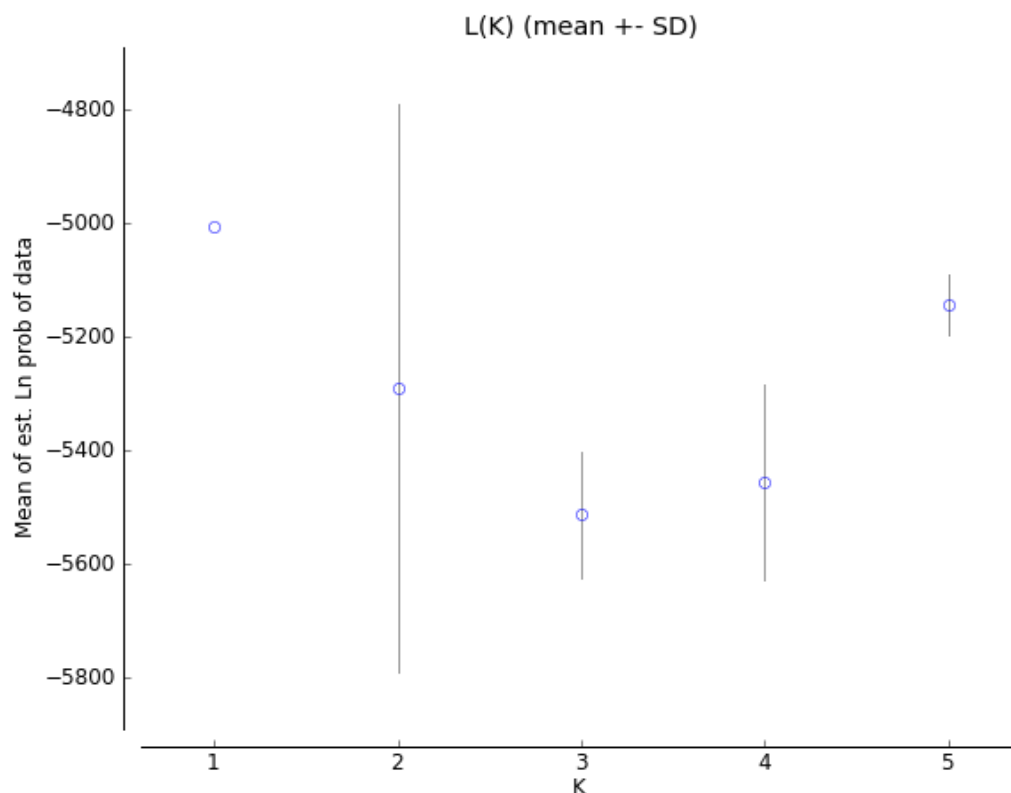

Fig. S1.1 The STRUCTURE analysis showed the highest probability and the lowest variance for $K=1$ and hence indicated a single gene pool in the study region.

S2 Details on genetic markers

Table S2.1 Details on microsatellite loci, combinations in multiplex PCR mixes and labelling of forward primers.

Locus	Reference[a]	MultiplexMix/ PCR	Label
epi3	Santos-del-Blanco *et al.* 2012	1	NED
FRPP94	Mariette *et al.* 2001	1	FAM
ITPH4516_3	Mariette *et al.* 2001	1	PET
NZPR5448	Chagné *et al.* 2004	1	FAM
pEST2669	Steinitz *et al.* 2011	1	NED
B4F08	Guevara *et al.* 2005	2	FAM
PtTX3030	Elsik *et al.* 2000	2	PET
pEST8	Steinitz *et al.* 2011	[b]	FAM
PtTX3116	González-Martínez *et al.* 2004	[b]	VIC

[a] See reference list at the end of the Supporting Information.

[b] pEST8 and PtTX3116 were amplified individually each in a final volume of 10 μl containing 10 ng of template DNA, 1x PCR buffer, 0.2 mM of each dNTP, 1 U of GoTaq polymerase (Promega, Madison, WI), 0.2 μM of each primer, and 1.5 mM and 4.5 mM $MgCl_2$ for pEST8 and PtTX3116, respectively. For pEST8 a touch-down PCR protocol was used: denaturation at 94°C for 3 min followed by 10 cycles at 94°C (30 s), 60°-50°C (30 s) lowering the annealing temperature by 1°C/cycle, 72°C (30 s), followed by 25 cycles at 94°C (30 s), 50°C (30 s), 72°C (30 s), and a final step at 72°C for 10 min. PtTX3116 was amplified using PCR protocol 3 described in Steinitz *et al.* (2011).

Table S2.2 Characterization of nine SSR loci in the *Pinus halepensis* stands with >60 samples: N_A, number of alleles and F_{IS}, fixation index. F_{IS} is followed by significance levels from 10 000 permutations and after Bonferroni multiple test correction: n.s., not significant.

Locus	Missing data [%]	N_A	Allele size range [bp]	F_{IS} Serra Calderona	Sinarcas	Eslida
ALL LOCI		44		0.044 n.s.	-0.073 n.s.	-0.006 n.s.
NZPR5448	1.5	3	244-254	-0.231 n.s.	-0.333 n.s.	-0.419 n.s.
Epi3	0.5	3	219-222	0.162 n.s.	-0.027 n.s.	-0.121 n.s.
FRP94	0.0	3	143-151	0.283 n.s.	-0.003 n.s.	0.309 n.s.
pEST2669	0.0	4	140-146	0.181 n.s.	-0.049 n.s.	-0.044 n.s.
Itph4516	1	16	134-174	0.075 n.s.	0.003 n.s.	-0.042 n.s.
pEST8	0	3	147-151	-0.098 n.s.	-0.171 n.s.	0.253 n.s.
PtTX3116	0	2	131-137	0.020 n.s.	-0.171 n.s.	0.288 n.s.
PtTX3030	1.5	5	348-357	0.048 n.s.	0.103 n.s.	0.022 n.s.
B4F08	1	5	177-199	0.027 n.s.	-0.051 n.s.	-0.103 n.s.

S3 Coalescent simulation analysis of demographic history

The command-line version of DIYABC v2.0 (Cornuet *et al.* 2014) was used to simulate genealogies for SSR data sets according to demographic scenarios that comprised several periods with constant effective population size each, sampling demographic parameters from prior distributions. A log uniform prior distribution (10^{-3}, 10^5) was used for theta, $\theta = 2N_e\mu$, the population scaled mutation rate. The number of population size changes was taken from a Poisson distribution with parameter $\lambda = \log(2)$ (nine changes were used when values higher than nine were drawn) and time points (scaled to mutation units, $\tau = t\mu$) were taken randomly from a uniform distribution in the interval from 0 to 5. Summary statistics (Table S3.1) were computed from a total of one million simulated data sets and the 0.1% simulations with summary statistics closest to those observed in each sample stand was retained. Skyline plots were built using the posterior distributions of θ (median and 95% highest density probabilities) at 100 time points, following the approach described in Burgarella *et al.* (2012) using R version 2.15.0 (R Development Core Team 2012) with the abc package (Csilléry *et al.* 2012).

Table S3.1 Summary statistics calculated in *DIYABC* for simulated and observed data.

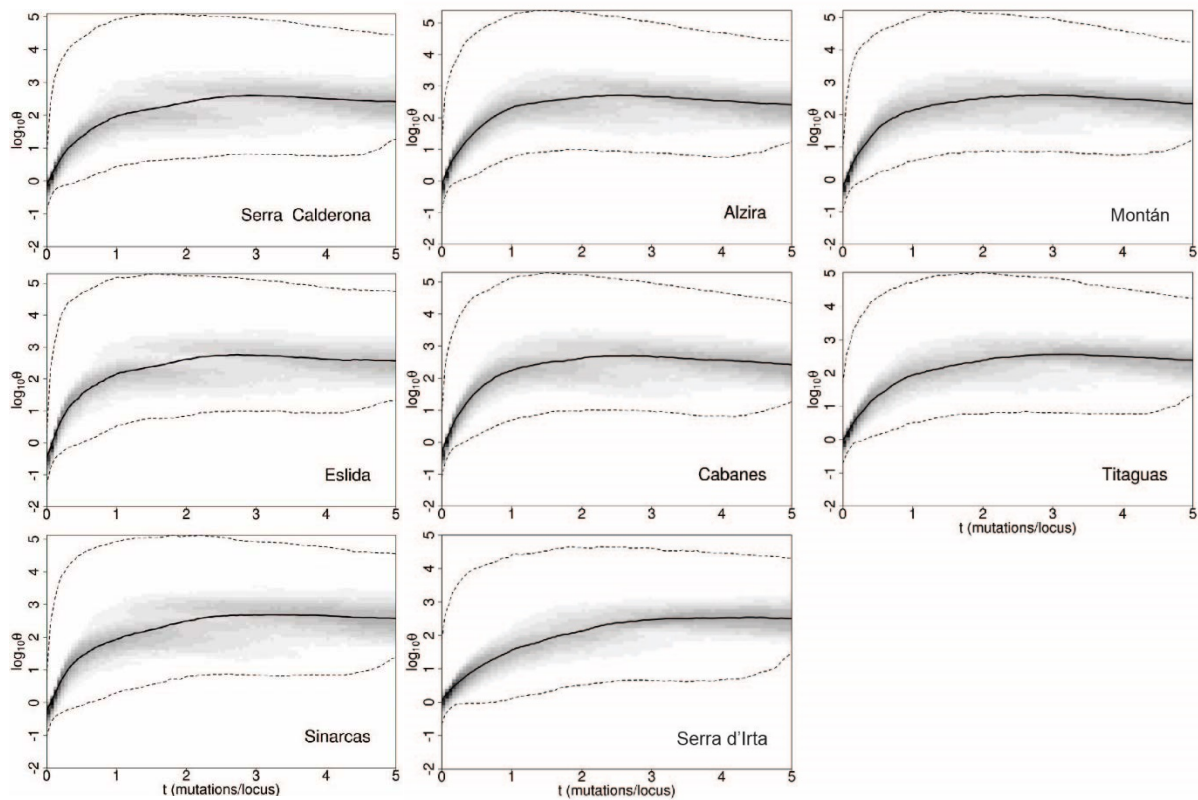|	Computation of all statistics at the population level
NAL	Mean number of alleles over loci
HET	Mean heterozygosity over loci
MGW	Mean Garza-Williamson statistic over loci
VAR	Mean allelic size (length in bp) variance over loci

Figure S3.1 Posterior density distribution of the mutation-scaled population size ($\theta=2N_e\mu$) as a function of time (as mutations/locus), estimated using Approximate Bayesian Computation for all *Pinus halepensis* stands. Black continuous lines represent

the medians of the posterior distribution and dotted lines the 95% highest posterior density intervals. Intensive study stands are the three to the left and the three LoFi stands are marked with a star.

S4 Spatially explicit simulations of genetic structure analysis

Spatially explicit simulations

To compare the power to detect and quantify SGS with both marker types (SSRs vs. SNPs), we conducted spatially explicit simulations of a reproducing and dispersing population comprising a grid of 100 x 100 individuals using the *simnew* program written by OJ Hardy (Heuertz *et al.* 2003; de-Lucas *et al.* 2009). Simulation parameters were chosen to be realistic for pines while using exaggeratedly restricted dispersal parameter values to enhance SGS build-up, because realistic values have previously resulted in weak and non-significant SGS (de-Lucas *et al.* 2009).

Simulations were conducted using nine SSRs to mimic the observed *P. halepensis* SSR data set and random sets of a variable number of SNP markers. Genotypes at generation 0 were assigned randomly to all individuals with allele frequencies mimicking those of a random pine stand. At each generation, 50% of the simulated trees were replaced by offspring resulting from dispersal according to Gaussian dispersal curves with an axial standard deviation of dispersal for seeds $\sigma_s = 3.77$ m, for pollen $\sigma_p = 22.72$ m and for genes $\sigma_g = 16.5$ m (see de-Lucas *et al.* 2009), allowing for 20% pollen immigration and 0% seed immigration per generation. SGS was allowed to build up for 64 generations, running 100 repetitions for each data type. At generations 2, 4, 8, 16, 32, 48 and 64, *b* and *Sp* were computed and the mean and standard deviation of *Sp* were compared across simulations for both marker types to determine the number of SNPs that yielded a similar standard deviation (and hence equal power for SGS detection) as the 9 SSRs used in *P. halepensis*.
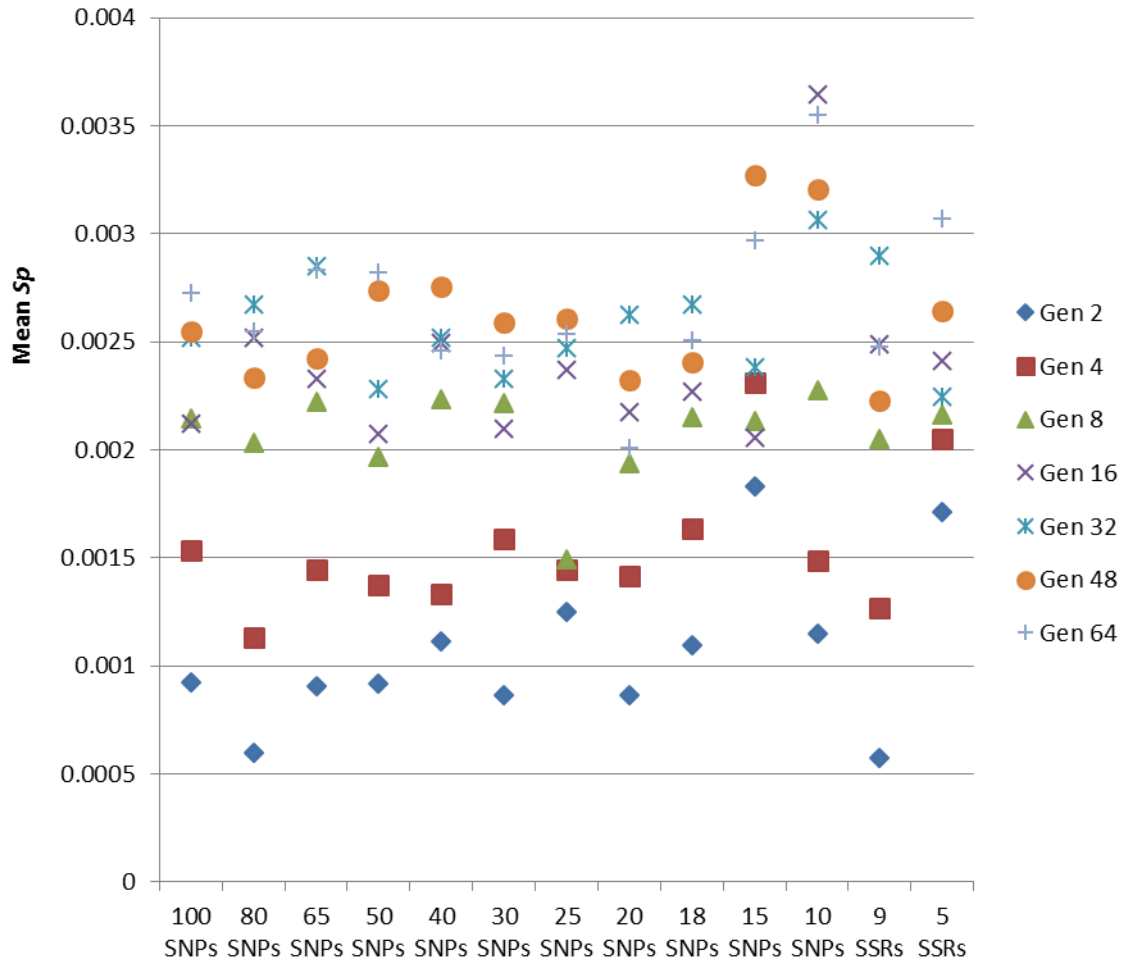
Fig. S4.1 Mean *Sp* values in simulated data sets. Spatial genetic structure builds up across generations and reaches stable values around generation 32. SNP and SSR data show similar mean *Sp* values.
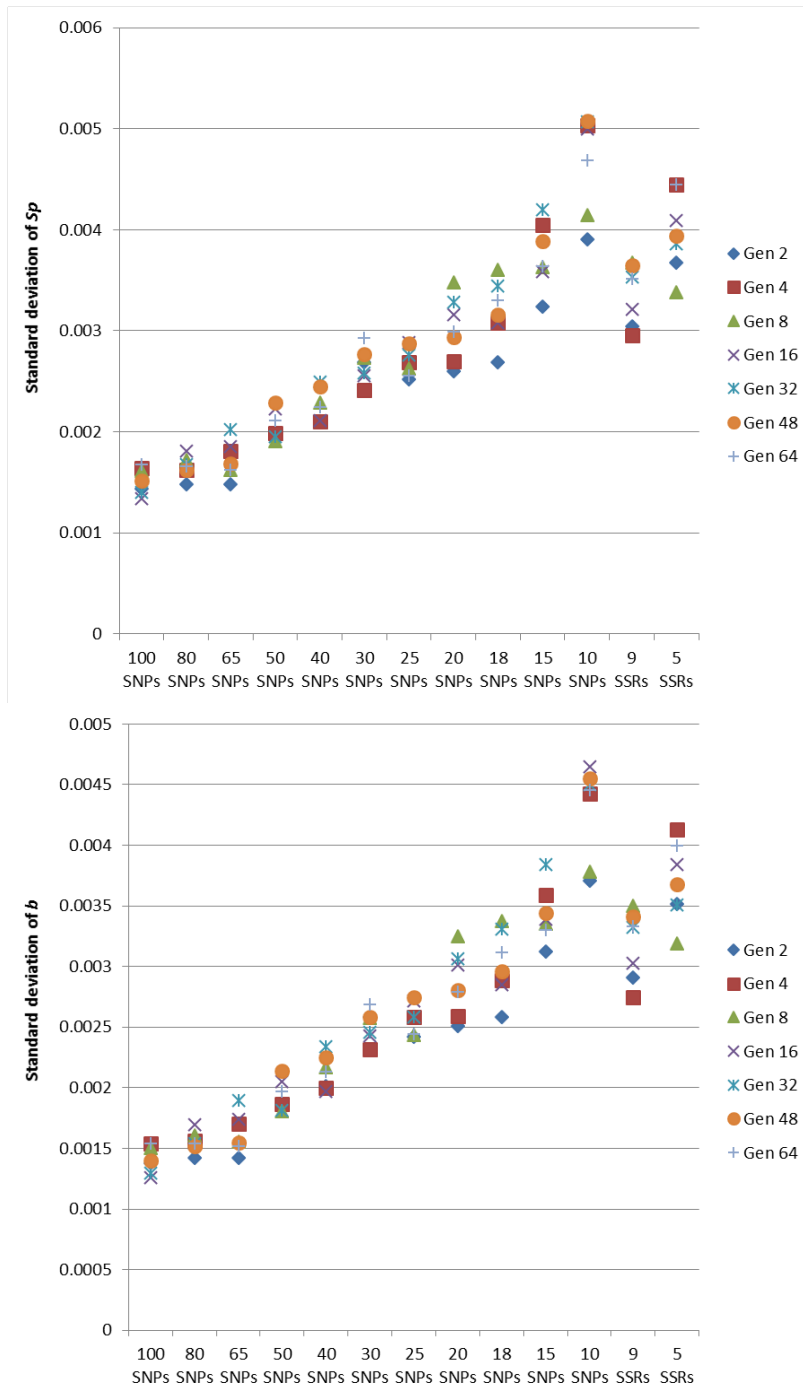
Fig. S4.2 Standard deviation of SGS estimates, expressed as standard deviation of *Sp* (above) or *b* (below), in simulated data sets. Simulated sets of 15 – 25 SNPs have similar standard deviation as SSR data sets in *P. halepensis* and, therefore, similar power to detect and quantify SGS.
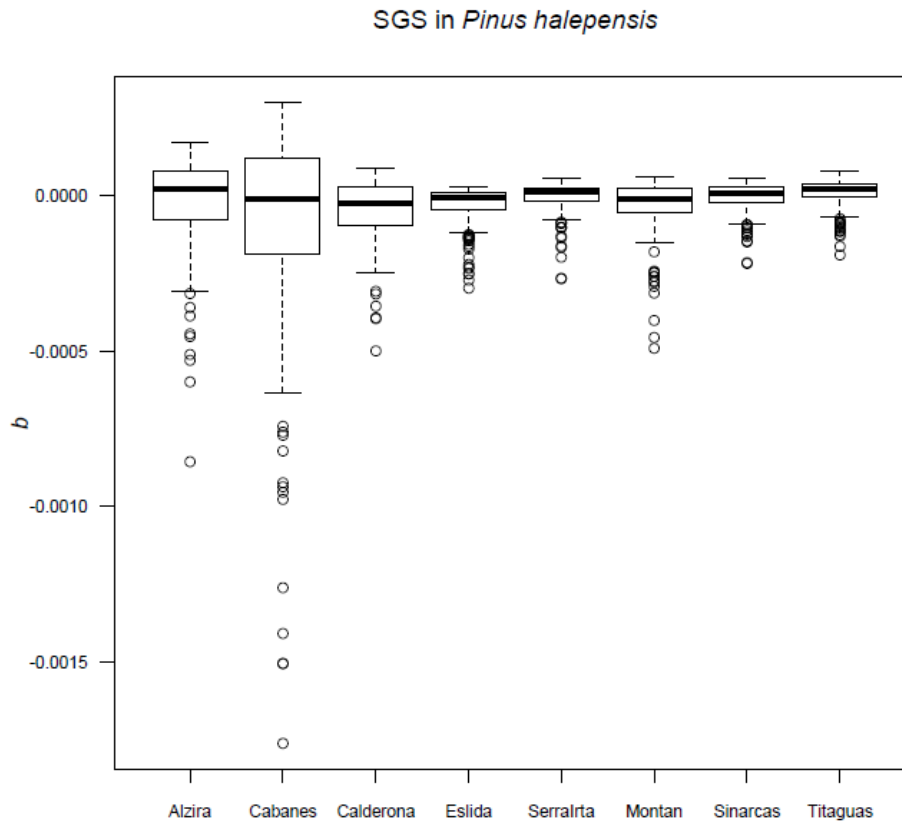
Fig. S4.3 Boxplot showing the distribution of single-locus SGS (regression slope *b*) and highlighting outlier loci. Whiskers extend to 1.5 times the interquartile range.

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