

# Analysis of spatial genetic structure in an expanding *Pinus halepensis* population reveals development of fine-scale genetic clustering over time

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## Abstract

We analysed the change of spatial genetic structure (SGS) of reproductive individuals over time in an expanding *Pinus halepensis* population. To our knowledge, this is the first empirical study to analyse the temporal component of SGS by following the dynamics of successive cohorts of the same population over time, rather than analysing different age cohorts at a single time. SGS is influenced by various factors including restricted gene dispersal, microenvironmental selection, mating patterns and the spatial pattern of reproductive individuals. Several factors that affect SGS are expected to vary over time and as adult density increases. Using air photo analysis, tree-ring dating and molecular marker analysis we reconstructed the spread of reproductive individuals over 30 years beginning from five initial individuals. In the early stages, genotypes were distributed randomly in space. Over time and with increasing density, fine-scale (< 20 m) SGS developed and the magnitude of genetic clustering increased. The SGS was strongly affected by the initial spatial distribution and genetic variation of the founding individuals. The development of SGS may be explained by fine-scale environmental heterogeneity and possibly microenvironmental selection. Inbreeding and variation in reproductive success may have enhanced SGS magnitude over time.

**Keywords:** microenvironmental heterogeneity, microsatellites, *Pinus halepensis*, population expansion, spatial autocorrelation, spatial genetic structure

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## Introduction

Spatial genetic structure (SGS), i.e. nonrandom spatial distribution of genotypes and alleles, usually in the form of within population fine-scale aggregation (Wells & Young 2002; Vekemans & Hardy 2004) has been described in various plant groups and affects various evolutionary and ecological processes (Heywood 1991; Ennos 2001; Vekemans & Hardy 2004). SGS can influence population dynamics and persistence by affecting levels of adaptation to microenvironmental variation (Epperson 1992), mating patterns (Young & Merriam 1994), intensity of density-dependent processes (Hamrick *et al.* 1993), effective population size and inbreeding and consequently progeny fitness

(Hamrick & Nason 1996; Schnabel *et al.* 1998). Knowledge of SGS is important for understanding population dynamics and may be utilized in forest and reserve management and planning (Epperson 1990b, 1992; Chung *et al.* 1998). Characterizing the change of SGS over time, particularly in expanding populations, may provide important insights, especially in light of processes such as global climate change, fragmentation and invasion; yet, this topic remains relatively unexplored.

It is likely that the factors which influence SGS in general, affect SGS also in expanding populations. We therefore summarize the major factors and suggest a new framework for studying the dynamics of SGS in expanding populations (Table 1). Limited gene dispersal can result in an excess of mating by proximity and consequently in isolation by distance within a continuous population (Wright 1943). The same factors are predicted to produce

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**Table 1** Summary of factors influencing SGS, their expected change over time or with increasing density, and its resulting effect on SGS

Factor	Expected change	Reference	Effect of expected change on SGS	Reference
Seed dispersal				
Wind driven seed dispersal*	Decrease	Guries & Nordheim (1984) Nathan <i>et al.</i> (2002a) Nathan & Katul (2005)	Increase	Suggested by Young & Merriam (1994)
Seed shadow overlap	Increase		Decrease	Hamrick <i>et al.</i> (1993) Hamrick & Nason (1996) Vekemans & Hardy (2004)
Inbreeding/selfing	Increase (nonrandom mating, fragmentation)	Wright (1946) Murawski & Hamrick (1991) Lowe <i>et al.</i> (2005)	Increase	Wright (1943) Linhart <i>et al.</i> (1981) Sokal & Wartenberg (1983)
Outbreeding/outcrossing	Increase (random mating)	Farris & Mitton (1984) van Treuren <i>et al.</i> (1993) Hartl & Clark (1997)	Decrease	Epperson & Allard (1989) Epperson (1990a)
Variation in reproductive success	Unknown		Increase if present	Schnabel <i>et al.</i> (1998)
Spatial aggregation of reproductive individuals	Decrease	Kenkel (1988) Bleher <i>et al.</i> (2002) McDonald <i>et al.</i> (2003)	Decrease	Doligez <i>et al.</i> (1998)
Generation overlap	Increase		Increase	Doligez <i>et al.</i> (1998)
Microenvironmental selection	Unknown		Increase if present	Epperson (1990b, 1992) Ennos (2001)
Density-dependent processes	Increase	Harper (1977) Augsburger (1983)	Decrease	Hamrick <i>et al.</i> (1993) Hamrick & Nason (1996) Epperson & Alvarez-Buylla (1997)

\*For primarily wind-dispersed species.

genetic subdivision on a local, within population scale as well (Sokal & Wartenberg 1983; Epperson 1990a; Heywood 1991). Many field studies have indicated restricted gene flow as the major factor affecting SGS formation (Heywood 1991; Ennos 2001; González-Martínez *et al.* 2002; Wells & Young 2002). Microenvironmental selection (Epperson 1990b, 1992; Ennos 2001) and historical events (Chung *et al.* 1998; Ennos 2001; Marquardt & Epperson 2004) may also lead to SGS formation.

Several factors that affect SGS magnitude are expected to vary over time and as adult density increases (Table 1). Vekemans & Hardy (2004) compared SGS in low-density vs. high-density populations and found stronger SGS in low-density populations, although gene dispersal distances were higher in the latter. In densely vegetated landscapes, wind-dispersed species may exhibit stronger SGS than in open landscapes, since seed dispersal may be reduced due to less intense winds in denser and taller vegetation (Nathan *et al.* 2002a; Nathan & Katul 2005) and disruption of seed autorotation by collision with trees (Guries & Nordheim 1984). The potential for outcrossing (a reducing factor) with newly established planted and natural populations might increase over time, although fragmentation may reduce outcrossing (Lowe *et al.* 2005). Inbreeding may increase SGS and is expected to be higher in small popula-

tions and inversely correlated with plant density if mating within neighbourhoods is random (although see Morgante *et al.* 1991). However, the relationship of inbreeding with density and its change over time may be complicated or altered by nonrandom mating, variation in reproductive success and SGS itself (Table 1).

The spatial pattern of reproductive individuals may also change over time (Table 1). Spatial aggregation is predicted to increase the magnitude of SGS in comparison to random or regular distributions (Doligez *et al.* 1998). In general, a shift from aggregation to regularity with increasing density is expected due to competition processes (Table 1). In addition to evaluating its effect on SGS, spatial pattern analysis can help infer ecological processes and forces such as microenvironmental heterogeneity (McDonald *et al.* 2003), dispersal patterns (Bleher *et al.* 2002) and competition (Kenkel 1988). It is therefore important to accompany SGS analyses with exploration of the spatial pattern and its corresponding dynamics over time.

The temporal component of SGS has been studied mostly by analysing different age cohorts or life stages. Generally, SGS in younger age classes is stronger than in older age classes, most likely due to density-dependent processes that result in thinning (Hamrick *et al.* 1993; Hamrick & Nason 1996; Epperson & Alvarez-Buylla 1997). The opposite

pattern has been found, presumably due to historical events or local selection (Kalisz *et al.* 2001). Simulation studies predict that SGS will develop quickly under isolation by distance and that old populations are expected to have stronger SGS than recently established populations (Sokal & Wartenberg 1983; Epperson 1990a, 2005). An increase in generation overlap may also increase the magnitude of SGS (Doligez *et al.* 1998). Chung *et al.* (2000) reported that the lack of SGS might be due to recent population establishment and random distribution of the initial seedlings. Hossaert-McKey *et al.* (1996) suggested that two opposing processes affected SGS in a recently established population: on the one hand, a decline in SGS due to outcrossing among founder genotypes; on the other hand, an increase due to mating between relatives.

In this study, our goal was to investigate the change in SGS over time in an expanding population. We analysed the change of SGS in reproductive individuals over time as well as the temporal change in several potential influencing factors. To our knowledge, this is the first empirical study to analyse the temporal change in SGS by following a single cohort over time rather than by analysing different cohorts (single-time approach). We selected an expanding *Pinus halepensis* population and applied methods of air photo analysis, tree-ring dating, molecular marker analysis and spatial pattern analysis. This enabled us to assess the effect of different factors on SGS and in specific cases (e.g. genetic diversity and spatial pattern) to evaluate their temporal change.

Based on published literature on SGS (Table 1) and preliminary knowledge on the specific study species and site, we formulated the following hypotheses regarding the temporal change of SGS in an expanding *P. halepensis* population:

- 1 In the early stages of population expansion, fine-scale SGS would develop due to the low density of reproductive individuals and limited seed dispersal.
- 2 Over time and with increasing tree density, SGS magnitude would decrease due to one or more of the following reasons: (i) greater seed shadow overlap; (ii) intensified density-dependent processes; and (iii) a shift from spatial aggregation of reproductive individuals to spatial randomness.
- 3 Alternatively, SGS magnitude would increase as a result of reduced seed dispersal distances due to less intense winds and increased density.

## Materials and methods

### Study species

*Pinus halepensis* Miller (Aleppo pine) is the most widely distributed pine in the Mediterranean basin (Barbéro *et al.*

1998; Quézel 2000). It has been widely introduced throughout the world and is considered one of the most invasive pine species and a very successful colonizer (Barbéro *et al.* 1998; Richardson 2000). It is very drought resistant and grows especially well on dry rocky limestone soils (Barbéro *et al.* 1998; Quézel 2000).

Pollination occurs in spring (March–April), fertilization about 1 year later and seed dispersal begins 3 years after pollination (Thanos & Daskalaku 2000). The species is an obligate seeder and bears cones at an early age—7–10 years (Thanos & Daskalaku 2000). Seeds are winged and primarily wind-dispersed, with no evidence of secondary dispersal by wind or animals (Nathan & Ne'eman 2000). Estimated seed dispersal curves are typically leptokurtic and most seeds do not travel > 20 m from the canopy edge (Nathan *et al.* 1999, 2000, 2001; Nathan & Ne'eman 2000, 2004).

Density-dependent processes, e.g. seed predation (mainly by ants and rodents) and seedling competition, have been shown to play an important role in Aleppo pine recruitment (Nathan & Ne'eman 2000, 2004). Predation rates were found to decline with increasing distance from trees and seed-to-sapling survival probabilities increase drastically with increasing distance from adult trees and with decreasing density (Nathan *et al.* 2000; Nathan & Ne'eman 2004).

### Study site

The study site is a 60 ha plot at Mt Pithulim in the Judean Hills, Israel (31°45'N, 35°04'E; altitude 628 m above sea level). The site inhabits a presumably native naturally regenerated Aleppo pine population, with no documented or field evidence of planting, cutting or fires (Nathan 2004). The site was relatively isolated from other Aleppo pine populations for a long period. The closest natural stand is located ~5.5 km from the focal stand. Earliest planted stands are located 1.5 km to the southwest (since 1940s) and 1 km to the east (since 1960s). Five trees, clearly visible in air photos as early as 1944, are located in the focal stand (Fig. 1) and are presumably the ancestors of the current population (> 2000 individuals). Air photos of the study site in approximately 10 years intervals (1944–2000) were obtained from the survey of Israel (Israel Ministry of Construction). Grazing, presumably by livestock herds, was intense in the site until 1948. Parent rocks are Cenomanian, including limestone, dolomite and marl and chalks. Soil is mostly shallow light rendzina. Surrounding vegetation is a mixture of dense to open scrubland. Further details can be found in Nathan *et al.* (1999) and Nathan (2004).

### Sampling strategy and field measurements

We focused on the initial stages of population expansion (reproductive population between 1958 and 1973). Air

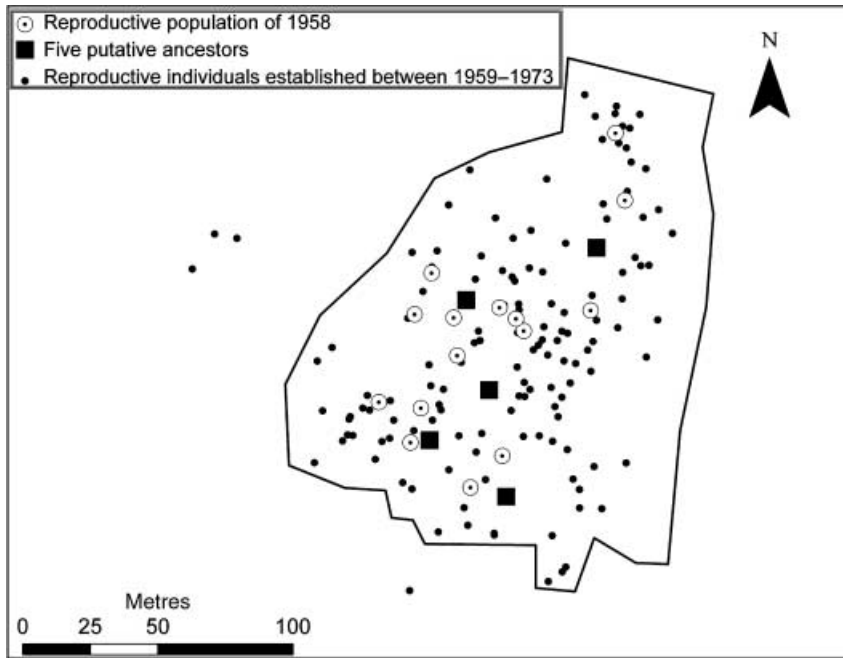


Fig. 1 Distribution of the sampled individuals in the focal stand (delineated by the solid line).

photos from early years (1949, 1956, 1963 and 1974) were analysed using a computer based stereoscopic image analysis module (Ofek Aerial Photography Ltd). Suspected tree locations were visited on site and when pines were found, they were tagged and their coordinates were recorded using a differential GPS unit (Trimble, GeoExplorer CE) coupled with a laser rangefinder (Advantage, Laser Atlanta). We sampled a total of 256 individuals.

Mortality processes in forest trees in general (Epperson 1992), and in Aleppo pine in particular (Nathan & Ne'eman 2004), occur primarily at early stages of recruitment. Therefore, and since maturity is reached after 7–10 years, reproductive population is assumed to consist of all individuals germinated at least 10 years earlier. For example, all trees estimated to germinate up to 1950 (see Dendrochronology section), are considered the reproductive population of 1960. This approach does not include mortality of reproductive individuals, which we found to be very low in the site (D. Troupin, unpublished).

#### Dendrochronology

We extracted core samples from each tree at 0.5 m above ground using a manual increment borer (Haglöf). Cores were not taken from a few trees with stems diverging close to the ground. Cores were glued into wooden mounts, sanded and scanned using a standard scanner (HP OfficeJet G85). Core photos were imported to ARCMAP software (ESRI ARCVIEW 8.3) and tree-ring widths were measured digitally. We estimated growth curves for each tree-ring width series. Tree-ring series were standardized by dividing

the observed ring width by the growth curve estimate, in order to remove variance in ring widths, which could be caused by nonclimatic factors and growth characteristics (Stokes & Smiley 1968; Schweingruber 1988). Skeleton plots were constructed for each individual and crossdated by comparison to a master plot (Stokes & Smiley 1968). We verified cross-dating quality using the program COFECHA (Holmes 1983), which is used to perform quality control on tree-ring measurements and indicates possible dating errors and measurement problems. Successfully dated samples were added to the master chronology. Samples with potential problems or errors were carefully re-examined and corrected if problems were identified. Most samples (92%) were successfully dated and their estimated germination year was determined.

#### DNA extraction and microsatellite analysis

One-year fresh needles were collected from each individual and transferred in dry ice to storage in  $-80^{\circ}\text{C}$  until extraction. From ~30 individuals, closed ripe cones were collected and opened later in order to retrieve seeds. Seeds were stored at  $4^{\circ}\text{C}$ . Total genomic DNA was extracted from 1-year needles and seed mega-gametophytes. When using seeds, at least six mega-gametophytes were used for genotyping individuals. We extracted DNA using the DNeasy 96 plant kit (QIAGEN) and quantified all extracted DNAs using a fluorometer (Hofer DyNA Quant200). We scanned a sample of trees for polymorphism using 20 paternally inherited chloroplast microsatellites (Vendramin *et al.* 1996) and seven biparentally inherited nuclear micro-

satellites designed for *P. halepensis* (Keys *et al.* 2000). We also tested three microsatellites designed for *P. taeda* (González-Martínez *et al.* 2004) and 20 microsatellites designed for *P. pinaster* (Guevara *et al.* 2005). Polymerase chain reactions (PCR) were performed in a PerkinElmer model 9600 thermal cycler. Total reaction volume was 25  $\mu$ L, containing 0.2 mM of each dNTP, 2.5 mM of MgCl<sub>2</sub>, 0.2 mM of each primer, 10 $\times$  reaction buffer, 25 ng of template DNA, 1 U of *Taq* polymerase (Amersham Biosciences) and 1.0% of BSA. Amplification profiles for each marker are described in the respective publications. One of the two primers in each reaction was 5' Cy5 labelled. Amplified microsatellites of different expected sizes (2 or 3) were loaded simultaneously on 6% 20 cm long denaturing polyacrylamide gels (ReproGel Long Read Amersham Biosciences). Gels were run on an ALFExpress automated sequencer (Amersham Biosciences). Fragment sizing was performed using the FRAGMENT MANAGER version 1.2 conversion software (Amersham Biosciences). External and internal molecular standards of known base pair length (50–250 bp) and previously sized samples were used in conjunction with the software in order to size amplified fragments. A high proportion of samples were run at least twice.

#### Spatial pattern analysis

We analysed the spatial pattern of trees using Ripley's *K*-function (Ripley 1977, 1981). We performed the analyses on the reproductive population of each year between 1958 and 1973 (Fig. 2), using the software PROGRAMITA (Wiegand & Moloney 2004). PROGRAMITA implements a numerical approach by creating an underlying cell grid and enables defining irregularly shaped study sites, thus removing the

need for edge corrections. We determined grid cell size to be 2  $\times$  2 m. The area of the focal stand and points within it (Fig. 1) were used for these analyses. We present the results using the L-function (Besag 1977), a commonly used transformation that linearizes *K*(*r*) and stabilizes its variance (Ripley 1981). Assuming complete spatial randomness (CSR), *L*(*r*) = 0 for all *r*-values. Positive values indicate clumping whereas negative values suggest regularity.

We compared the spatial patterns to two different null models:

- 1 Complete spatial randomness (CSR) which is implemented as a homogeneous Poisson process (Diggle 1983), i.e. the intensity  $\lambda$  is constant over the study region. Any point of the pattern has an equal opportunity of occurring at any position in the study region.
- 2 A heterogeneous Poisson process which is suitable when the intensity is not constant throughout the study region (Wiegand & Moloney 2004). The constant intensity is replaced by an intensity function that varies with location and is estimated by a circular moving window ( $R = 20$  m). The occurrence of any point remains independent of that of any other.

The statistical significance of *K*(*r*) values was estimated by 95% confidence intervals generated by 1000 Monte Carlo simulations of the null models. Circle radii for the *K*(*r*) analysis increased by 2-m increments up to the maximum distance of 100 m.

#### Genetic diversity analysis

We calculated the following genetic diversity measures for the reproductive population between the years 1958 and

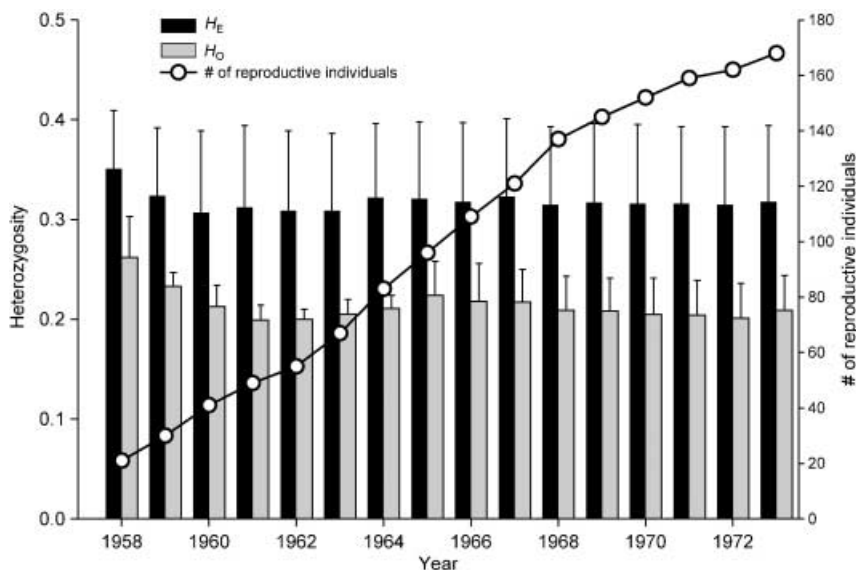


Fig. 2 Estimated size of reproductive population and mean ( $\pm$  SE) values of the corresponding observed heterozygosity ( $H_O$ ) and expected heterozygosity ( $H_E$ ) over the years 1958–1973. Means were calculated over four nuclear SSRs.

1973 (Fig. 2), using the GENALEX 6.0 software (Peakall & Smouse 2005): expected heterozygosity ( $H_E$ ), observed heterozygosity ( $H_O$ ) and the effective number of alleles ( $N_e$ ). The inbreeding coefficient within individuals relative to the population ( $F_{IS}$ ) (Weir & Cockerham 1984) and its significance were calculated using the GENEPOP 3.4 software (Raymond & Rousset 1995). Significant positive  $F_{IS}$  values indicate inbreeding (excess of homozygotes) or undetected null alleles, whereas significant negative values indicate excess of heterozygosity. Near zero values are expected under random mating. Significant deviation of  $F_{IS}$  from zero was tested by Hardy–Weinberg exact tests.  $F_{IS}$  across all nuclear loci was estimated using Fisher's method. All measures were calculated only for nuclear markers.

The temporal change of the diversity measures was evaluated by linear regressions (SPSS 12.0). In several cases where we estimated certain parameters for the whole reproductive population in consecutive years, the observations were not independent. We therefore performed Mantel tests in addition to the regression analyses. The null hypothesis that the observed change over time of certain parameters could have been obtained by any random arrangement of the observations in time was tested. Mantel tests (10 000 iterations) were performed using the POPTOOLS add-in for Microsoft Excel (Hood 2005).

### Spatial genetic structure analyses

We first used spatial autocorrelation methods for detecting SGS. Spatial genetic autocorrelation (Sokal & Oden 1978) measures the correlation in allelic or genotypic state between individuals separated by defined distances within the whole population. We used the multivariate autocorrelation procedure, which strengthens the spatial signal and reduces the allele to allele stochasticity and locus to locus noise (Smouse & Peakall 1999). We used the GENALEX 6.0 software (Peakall & Smouse 2005) for spatial autocorrelation analyses (see Smouse & Peakall 1999; Peakall *et al.* 2003, for detailed description).

The correlation coefficient  $r$  is bounded by  $-1$  and  $1$ , has a mean of zero when there is no autocorrelation and is closely related to Moran's  $I$ . Significant positive autocorrelation implies that individuals within a particular distance class are more genetically similar than expected by random (Ennos 2001). The statistical significance was tested by random permutations (1000 simulations) and 95% confidence intervals, generated by bootstrapping (1000 repeats). We performed the analyses on the reproductive population of each year between 1958 and 1973 (Fig. 2). We performed all analyses using both the even distance classes option (using 10 distance classes, 10 m each) and the even sample size option (testing various sample sizes).

We further quantified SGS using the ' $S_p$ ' statistic (Vekemans & Hardy 2004) that is less sensitive to the sampling design

used, and allows the comparison of SGS magnitude among different populations (Vekemans & Hardy 2004) and in our case between different years. We did not rely solely on ' $S_p$ ' since it strongly depends on the monotonous decrease of the kinship coefficients with distance (not true in our case) and relatively little is known about its statistical properties (Epperson 2005). ' $S_p$ ' reflects the decrease rate of the kinship coefficient with distance.  $S_p$  is calculated  $-\hat{b}_F/(1 - \hat{F}_{(1)})$  where  $\hat{F}_{(1)}$  is the mean kinship coefficient  $F_{(ij)}$  (Loiselle *et al.* 1995) between individuals in the first distance class and  $\hat{b}_F$  is the regression slope of the  $\hat{F}_{(r)}$  coefficients against the logarithm of the distance. We used 10 distance classes (10 m each). These statistics and their significance (10 000 permutations) were calculated using the software SPAGED1 (Hardy & Vekemans 2002).

### Test of sample size influence

Since the sample size increased over time (from 20 reproductive individuals in 1958 to 168 in 1973) we tested whether this change alone caused the temporal change of SGS. We randomly sampled 20 individuals from the reproductive population in different years (1960, 1966 and 1973), repeated this procedure 50 times for each year and calculated the correlation coefficient in the distance class 0–20 m and the  $S_p$  statistic for all repetitions. We then tested by linear regression whether these statistics changed over time.

## Results

### Temporal dynamics of population expansion

From within the sample of successfully dated trees (236 out of 256 individuals), a total of 168 individuals comprised the reproductive population in 1973 (estimated germination year = 1963, Figs 1 and 2). Our reconstruction of the population expansion over 30 years (1944–1973) confirms that only the five putative ancestors were found in the study site before 1944 (Figs 1 and 2). In the years 1944–1948, 16 trees that reached reproductive age germinated (Figs 1 and 2) indicating that the majority of trees we sampled germinated > 1948 (upon removal of the grazing pressure). During the 1960s, reproductive population size increased sharply (Figs 1 and 2).

### Temporal dynamics of spatial pattern

*Under complete spatial randomness.* In the earliest year analysed (1958, Appendix)  $L(r)$  values were within confidence intervals at all distances indicating that the spatial pattern was not significantly different from random. In the subsequent years (1959–1973) the observed pattern was relatively identical among the different years.  $L(r)$

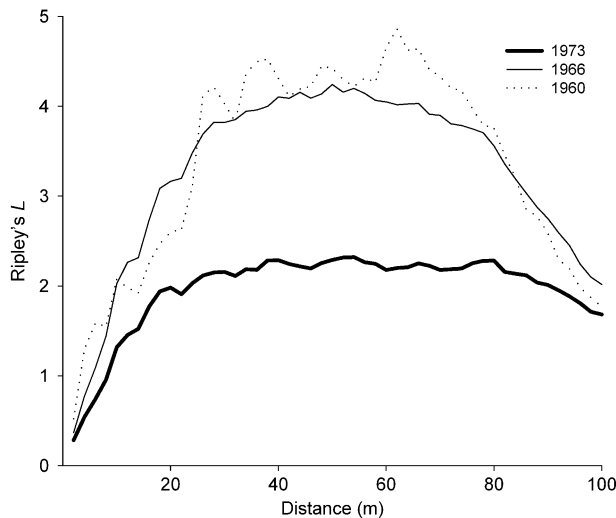


Fig. 3 Ripley's L-function in three different stages of population expansion.

increased linearly up to distances of 20–30 m, remained relatively constant up to 70–80 m and then decreased (Appendix). In the years 1959–1962  $L(r)$  values were within the confidence intervals in short distances (0–20 m) and significantly positive above these distances. Beginning in 1963,  $L(r)$  values were significantly positive for all distances > 6 m, indicating a more clustered spatial distribution than expected by random.  $L(r)$  values decreased over time (Fig. 3) indicating a weakening in aggregation.

*Under heterogeneous poisson process.*  $L(r)$  values were within confidence intervals in all years (not shown), indicating no significant deviation from the null hypothesis.

### Temporal dynamics of genetic diversity

Four nuclear loci and one chloroplast region exhibited polymorphism (Table 2) and were effectively scored in all sampled individuals. Another chloroplast region (Pt63718) was entirely monomorphic except for a rare haplotype found only in one individual. Within the 168 sampled individuals we found 55 distinct multilocus genotypes (combining nuclear and chloroplast markers). Within the five putative ancestors we found four distinct genotypes, i.e. apart from one genetically identical pair, they were genetically distinguishable. Apart from the aforementioned individual containing the rare chloroplast haplotype, all other 54 multilocus genotypes can be explained by the four original ones, i.e. apart from the alleles found in the five founding individuals, no new alleles were found.

Over time, the overall effective number of alleles ( $N_e$ , mean  $\pm$  SE =  $1.53 \pm 0.2$  in 1973, Table 3) and expected heterozygosity ( $H_E$ ) did not change whereas observed heterozygosity ( $H_O$ ) decreased significantly (Fig. 2, Table 3). The mean overall loci inbreeding coefficient ( $F_{IS}$ ) increased significantly over time (mean  $\pm$  SE =  $0.30 \pm 0.07$  in 1973, Table 3) and was significantly positive throughout all the period.

### Temporal dynamics of spatial genetic structure

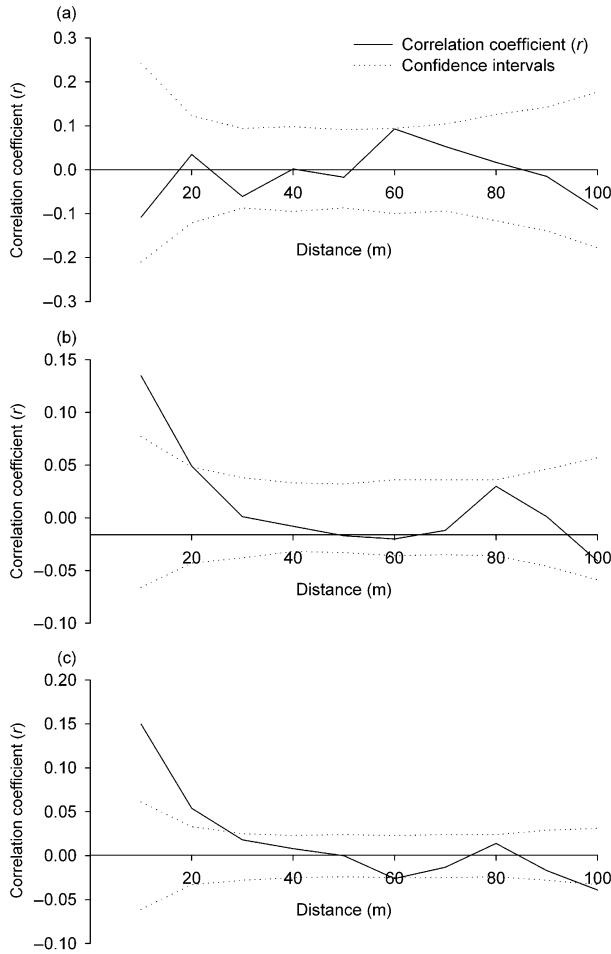
Results using even distance classes and even sample sizes were similar. We therefore present results of analyses using even distance classes, with the exception of the years 1958–1960, for which we present results of even sample size analyses that provided a minimum of  $n = 20$  comparisons per distance class. Thus, the first distance classes were 19 m, 13 m and 11 m and the second distance classes were 27 m, 18 m and 16 m for the years 1958, 1959 and 1960, respectively.

Table 2 List of the polymorphic microsatellite loci. N and CP indicate nuclear and chloroplast markers, respectively. Reference: a, González-Martínez *et al.* (2004); b, Keys *et al.* (2000); c, Vendramin *et al.* (1996)

Primer name	Primer sequence (5'–3')	Genome	No. alleles/size variants in population	Source reference
PtTX3107	F: AAACAAGCCACATCGTCAATC R: TCCCCTGGATCTGAGGA	N	2	a
PtTX3116	F: CCTCCCAAAGCCTAAAGAAT R: CATAACAAGCCTTATCTTACAGAA	N	2	a
PHAF01	F: TTCAGATATGGTCCATGGATG R: GATCACAATGTCAATATCGG	N	2	b
PHAF07	F: ATCAGCTTAGTAGGTCGCGC R: AGACACTAAAGGGGAGTCCG	N	3	b
Pt63718	F: CACAAAAGGATTTTTTTTCAGTG R: CGACGTGAGTAAGAATGGTTG	CP	2	c
Pt71936	F: TTCATTTGAAATACACTAGCCC R: AAAACCGTACATGAGATTCCC	CP	2	c

Parameter	Linear regression				Mantel test	
	Slope	Constant	P value	R <sup>2</sup>	ρ	P value
N <sub>e</sub>	-0.006	1.54	0.524	0.029	-0.093	0.230
H <sub>O</sub>	-0.0017	0.228	<b>0.040</b>	0.268	-0.310	<b>0.005</b>
H <sub>E</sub>	-0.0005	0.323	0.308	0.074	-0.151	0.154
F <sub>IS</sub>	0.008	0.196	<b>&lt; 0.001</b>	0.851	0.800	<b>&lt; 0.001</b>

**Table 3** Results of linear regressions and Mantel tests for parameters of genetic diversity over time. Dependent factor in all analyses is the genetic diversity parameter. Independent factor is time. Significant values in bold



**Fig. 4** Results of spatial autocorrelation analysis for the years 1960 (a), 1966 (b) and 1973 (c). Solid lines represent the correlation coefficient (*r*) and dashed lines represent the confidence intervals.

In the years 1958–1965 we found no significant autocorrelation in the short distances (0–30 m) (Fig. 4a). Beginning in 1966 (Fig. 4b, c), significant positive autocorrelation was found in the first and second distance classes (10 m and 20 m, respectively). During this period (1966–1973) we also found very weak positive autocorrelation in the 80 m distance class (in 1968–1970) and very weak negative autocorrelation in the 60 m and 100 m distance classes

(1968–1973). Over time the correlation coefficient (*r*) of the first and second distance classes as well as the *Sp* values increased significantly (Fig. 5a–c, respectively). The positive correlations were confirmed by the results of the Mantel tests ( $\rho = 0.661, P < 0.001, \rho = 0.308, P = 0.004$  and  $\rho = 0.792, P < 0.001$  for first and second distance class correlation coefficients and the *Sp* statistic, respectively).

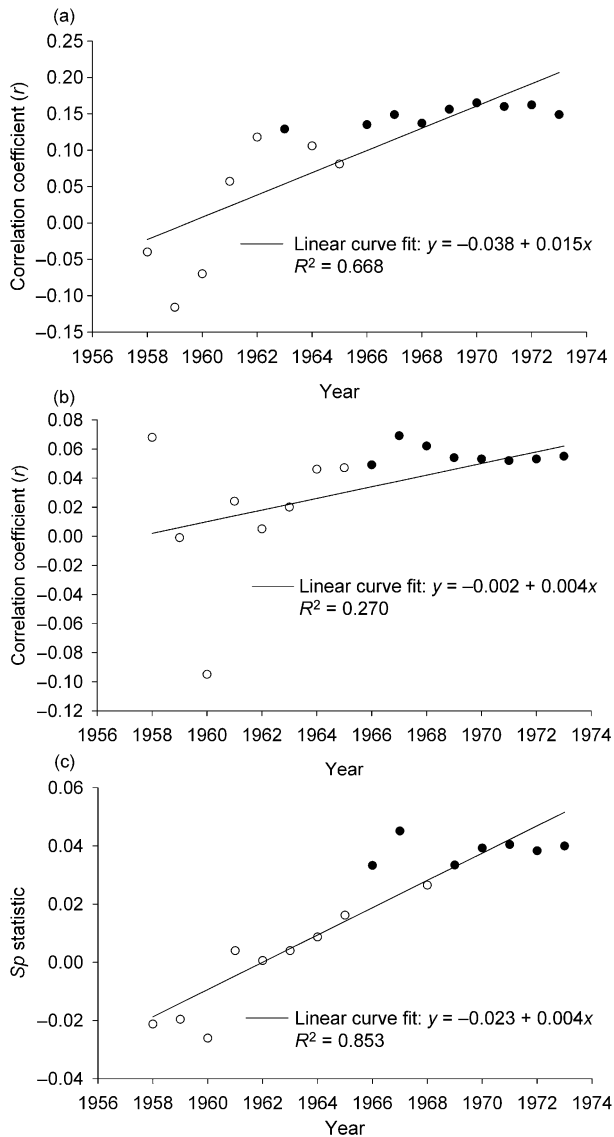
*Sample size influence*

Values of the correlation coefficient of the distance class 0–20 m obtained by randomly sampling 20 individuals (50 repeats for each time stage) significantly increased over time ( $R^2 = 0.14, P_{\text{slope} = 0} < 0.001$ ). The *Sp* statistic increased as well, however, this increase was not significant ( $R^2 = 0.15, P_{\text{slope} = 0} = 0.079$ ). *R*<sup>2</sup> values obtained in this procedure were relatively low due to the scatter generated by randomly sampling a small number of individuals for each time stage. Overall, these results indicate that the increase in SGS magnitude detected over time is not merely a result of the increase in sample size.

**Discussion**

Starting with five reproductive individuals in the 1940s, the Aleppo pine population of Mount Pithulim has expanded to > 2000 trees inhabiting the site today. Focusing on the first three decades of this expansion process, we found, as expected, a gradual increase over time in reproductive population size and density. In this first study of the dynamics of SGS in successive cohorts of an expanding tree population, we found no SGS in the early stages; yet, fine-scale genetic clustering developed over time and in later stages we found significant SGS. The processes underlying the development of SGS and the increase in its magnitude cannot be determined directly by pattern analysis; nevertheless, we do assess the contribution of several potentially influencing factors. We first discuss several factors that had no apparent effect on the development and increase in SGS in our stand, namely limited seed dispersal, seed shadow overlap, the spatial pattern of reproductive individuals and density-dependent processes. We then propose and discuss other factors, namely inbreeding, variation in reproductive success and selection in response





**Fig. 5** Increase over time of first (a) and second (b) distance class correlation coefficients and the  $S_p$  statistic (c). Solid lines represent linear curve fits ( $R^2 = 0.668$  and  $P_{\text{slope}=0} < 0.001$ ,  $R^2 = 0.270$ ,  $P_{\text{slope}=0} = 0.038$  and  $R^2 = 0.853$ ,  $P_{\text{slope}=0} < 0.001$  for first distance class, second distance class and the  $S_p$  statistic, respectively). Open and closed circles represent nonsignificant and significant values of the correlation coefficients and the regression slopes of the kinship coefficients against the logarithm of the distance, respectively.

to microenvironmental heterogeneity as possible causes of the development of SGS in our population.

Since the five presumed ancestors were relatively distant from each other and genetically distinguishable (apart from one pair), we hypothesized that this initial distribution along with limited seed dispersal would result in genetic clustering of their progeny (Hypothesis 1). We found, however, no SGS in the earliest stages investigated. The SGS analyses using even sample sizes and the test of

sample size influence we performed suggest that sample size did not influence the results. We suggest the random distribution of genotypes could be the result of density-dependent grazing. If the grazing process that dominated the site acted mainly on seedling clusters (Senft *et al.* 1987; Adler *et al.* 2001), it is possible that most of the reproductive individuals that germinated before 1948 were not located in proximity to their sources but randomly in relation to their sources.

Significantly positive SGS of the reproductive population was found beginning in 1966 and its magnitude increased over time as indicated by the increase of the correlation coefficient in the short distance classes ( $< 20$  m) and by the increase of the  $S_p$  statistic. The observed structure, whether random at first or significant but relatively weak in later stages, is typical for adult age classes of conifers and wind-dispersed species (Epperson & Allard 1989; Parker *et al.* 2001; Marquardt & Epperson 2004; Gapare & Aitken 2005). The development of SGS in our case matches the findings of simulation studies of isolation by distance. Such studies have found that when the initial distribution of genotypes is random, the degree of spatial autocorrelation quickly increases in the initial generations (Sokal & Wartenberg 1983; Epperson 1990a, 2005). It is also predicted that SGS will be stronger in old populations than in recently established ones due to generation overlap and increased kinship between mates (Doligez *et al.* 1998).

Aleppo pine seed dispersal estimates obtained in the same study site using mechanistic wind dispersal models (Nathan *et al.* 1999, 2001; Nathan & Ne'eman 2004) indicate that dispersal is highly restricted to distances  $< 20$  m, even under extremely windy conditions. These estimates may explain the clustering and its scale ( $< 20$  m) and support the role of limited seed dispersal as the main cause of SGS. However, establishment patterns (effective seed dispersal), obtained by parentage analysis (Troupin 2005) contradict this explanation. These complementary results indicate that successful establishment events occurred mostly at distances of 20–70 m from their source, i.e. effective dispersal distances were not as limited as seed dispersal. Thus, although seed dispersal is largely limited to the 20 m near the source, the subsequent establishment is not restricted to these distances (minimal effective dispersal distance = 11 m,  $n = 47$ , mean  $\pm$  SE =  $106 \pm 18$  m, Troupin 2005).

As tree density increases, wind intensities within a forest are predicted to decrease (Nathan *et al.* 2002a; Nathan & Katul 2005). Theoretically, this should result in reduced seed dispersal and consequently in an increase in SGS magnitude (Hypothesis 3); however, even if dispersal distances decreased over time, we found no indication of a similar decline in effective dispersal distances (Troupin 2005). Similarly, although increasing density is expected to result in higher seed shadow overlap and intensification of density-dependent processes and consequently reduced

SGS (Hypothesis 2), the increase in SGS suggests no strong influence of these factors.

The temporal dynamics of the spatial pattern of trees does not explain the increase in SGS magnitude (Hypothesis 2). Spatial aggregation is expected to increase SGS (Table 1); although we found that the reproductive individuals were spatially aggregated, this aggregation decreased over time. Spatial aggregation depends on various factors including population age, site characteristics and species life history (Aldrich *et al.* 2003; McDonald *et al.* 2003). Since there is no evidence of fires or human activity in the site, it is likely that the spatial aggregation was the result of the initial distribution of trees, which was probably determined by the arrival and establishment pattern of the earliest individuals. This distribution could have also been affected by livestock grazing, which was dominant in the site < 1948 (e.g. Aldrich *et al.* 2003). A shift over time from aggregation to a uniform/random dispersion is often related to density-dependent processes (Table 1). In an expanding population as ours, the shift towards randomness is more likely to have been due to the increasing tree density and relatively long distances of effective seed dispersal events. Bleher *et al.* (2002) showed that a few generations are enough for the initial spatial pattern to dissolve and that a highly clumped pattern could disappear within one generation if dispersal distances or plant density were high enough.

The fact that the heterogeneous Poisson null model was not rejected, suggests that there was variation in the spatial pattern intensity over the study site, i.e. in some areas tree density was higher than in others. This may indicate there is microenvironmental heterogeneity or a patchy distribution of favourable establishment sites, which could also account for spatial aggregation (Clark *et al.* 1999; Maestre *et al.* 2003). This corroborates independent evidence of high microenvironmental heterogeneity of topographic and edaphic factors in the study site (Nathan 2004; Svoray & Nathan 2005). Microenvironmental heterogeneity with respect to Aleppo pine recruitment and establishment requirements also may have been a key factor in the development of SGS. Selection in response to this fine-scale heterogeneity and/or variation in the level of adaptation of individuals to it could have resulted in SGS formation (Heywood 1991; Epperson 1992). If there was indeed fine-scale microenvironmental heterogeneity and establishment of individuals with different genotypes was microhabitat dependent, i.e. certain genotypes were preferred in certain microenvironmental conditions, then the increasing density of certain genotypes in distinct patches may have been the cause of increase in SGS. Microsatellites are generally considered selectively neutral; however, in our case of a population that expanded from a limited number of founding individuals, levels of linkage disequilibrium might be high and therefore markers might be associated with linked fitness loci (Hansson & Westerberg 2002).

Inbreeding and outcrossing have opposite effects on SGS magnitude (Table 1). We found that inbreeding ( $F_{IS}$ ) increased over time and was relatively high in comparison to other studies (Parker *et al.* 2001; Marquardt & Epperson 2004; Vekemans & Hardy 2004). This trend corresponds to the increase in SGS. In general, compared to other studies on conifers and wind-dispersed species, genetic diversity was low (Thomas *et al.* 1999; González-Martínez *et al.* 2002; Marquardt & Epperson 2004). High inbreeding and low genetic diversity were expected due to the small number of population founders (founder effect) as well as low levels of outcrossing (Murawski & Hamrick 1991; Ellstrand & Elam 1993; Robledo-Arnuncio *et al.* 2004).

A positive relationship between inbreeding and population size and/or density has been found in other studies (e.g. Luijten *et al.* 2000) and could be related to SGS, i.e. the spatial proximity of genetically related individuals and mating among them (Linhart *et al.* 1981; Thomas *et al.* 1999). In turn, this increase also could have positively affected SGS magnitude (Wright 1943; Sokal & Wartenberg 1983; Epperson 1990a). Lower wind intensities and denser landscape could have resulted in reduced pollen dispersal distances and consequently in excess of mating by proximity related individuals (Antonovics & Levin 1980). A higher proportion of mating events among related individuals could occur also due to increasing generation overlap (Doligez *et al.* 1998).

Variation in individual reproductive success also could have enhanced SGS formation and its magnitude (Dow & Ashley 1996; Schnabel *et al.* 1998). Over time and as population size increases, such variation may decrease due to more mating opportunities. Parentage analysis results indicate marked variation in individual reproductive success in the time period studied, although characterizing the change of this variation over time was not possible due to sample size considerations (Troupin 2005).

We found little evidence of gene flow from other populations (the rare chloroplast haplotype at Pt63718), probably due to the population's relative isolation in the early stages (1940s) of its expansion (the nearest source for outcrossing at the time was located 1.5 km away). Although our findings suggest otherwise, more extensive external gene flow cannot be excluded completely considering that both pollen and seeds of wind-dispersed conifers are capable of long-distance dispersal (Campbell *et al.* 1999; Nathan *et al.* 2002b). The low level of genetic diversity found in our population probably reduced the possibility of detecting such events.

## Conclusions

The SGS was strongly affected by the initial spatial distribution and genetic variation of the founding individuals. Limited seed dispersal was apparently not the cause of SGS formation. An alternative explanation for the



development of SGS might be fine-scale environmental heterogeneity and perhaps microenvironmental selection. Mating patterns (inbreeding) and variation in reproductive success may account for the increase in SGS magnitude over time. An in-depth analysis of the fine-scale environmental heterogeneity in the site is required. Demonstration of such heterogeneity and investigation of its correlation with the distribution of genetic variation should help confirm or reject the role of microenvironmental selection in SGS development. Field and greenhouse experiments examining the success of different genotypes and heterogeneity of microenvironmental conditions are planned in the future. Analyses of the surrounding populations and contemporary patterns are also in need. Understanding the change in SGS over time is complicated due to the multiple factors involved and the difficulty in disentangling their influence. Future research of SGS temporal dynamics should include the construction of suitable null models. Such models should incorporate major factors (e.g. seed dispersal, initial distribution of individuals, density and outcrossing) and will be indispensable in understanding the dynamics of SGS and determining the relative importance of its influencing factors.

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These results are part of David Troupin's MSc thesis on the seed dispersal and genetic structure in an expanding Aleppo pine population. Ran Nathan's Movement Ecology Laboratory at the Hebrew University of Jerusalem investigates various aspects of organism movements and their interactions with real-life environmental heterogeneity, including seed dispersal by wind and by animals, animal foraging and bird migration. GG Vendramin is a senior researcher of the National Research Council of Italy whose main interest is in phylogeography and in population and conservation genetics of tree species.

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**Appendix**

Ripley's L-function (solid lines) for the years 1960 (a), 1966 (b) and 1973 (c). Dotted lines represent confidence intervals for the CSR null model

