

# Fine-scale spatial patterns and genetic structure of two co-occurring plant congeners: fingerprints of coexistence?

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**Running headline:** Spatial genetic structure of congeneric plants

**Keywords:** clonal growth, community genetics, congeners, ISSR, genetic distance, Mantel correlogram, plant-plant interactions, spatial genetic structure, *Thymus*

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## Summary

- The spatial distribution of individual plants within a population and its genetic structure is usually determined by several factors, like seed dispersal, reproduction mode or biotic interactions. So far, the role of interspecific interactions in shaping the spatial genetic structure of plant populations remains largely unknown.
- Species with a common evolutionary history like congeners are known to interact more closely with each other than unrelated species due to the greater number of traits they share. We hypothesize that plant interactions may be shaping the fine genetic structure of closely related congeners.
- We used spatial statistics (georeferenced design) and molecular techniques (ISSR markers) to understand how two closely related congeners, *Thymus vulgaris* (a widespread species) and *Thymus loscosii* (a narrow endemic) interact at the local scale. Specific cover, number of individuals of both study species, together with several community attributes were measured in a 100 m<sup>2</sup> (10 x 10 m) plot.
- Both species showed similar levels of genetic variation, but they differed in their spatial genetic structure. *T. vulgaris* showed spatial aggregation but no spatial genetic structure, while *T. loscosii* showed spatial genetic structure (positive genetic autocorrelation) at short distances. The spatial pattern of *T. vulgaris*' cover showed significant **dissociation** with that of *T. loscosii*. The same was true between the spatial patterns of the cover of *T. vulgaris* and the **abundance** of *T. loscosii* and between the **abundance** of each species. Most importantly, we found a correlation between the genetic structure of *T. loscosii* and the abundance of *T. vulgaris*: *T. loscosii* plants were genetically more similar when they were surrounded by a similar number of *T. vulgaris* plants.

- *Synthesis.* Collectively, our results reveal spatially complex genetic structures of both congeners at small spatial scales. The negative association among the spatial patterns of the two species and the genetic structure found for *T. loscosii* in relation to the abundance of *T. vulgaris* suggest that competition between the two species may account for the presence of adapted ecotypes of *T. loscosii* to the abundance of a competing congeneric species. This suggests that the presence and abundance of close congeners can influence the genetic spatial structure of plant species at fine scales.

10 **Keywords:** spatial genetic structure, congeners, plant-plant interactions, clonal growth, *Thymus*, ISSR, community genetics

## Introduction

Plant populations are not random assemblages of genotypes but are structured in space and time at different scales, from regions to populations and from populations to local groups of neighbor plants (Loveless & Hamrick 1984; Escudero, Iriondo & Torres 5 2003). The spatial arrangement of individual plants within a population is determined by a diverse array of processes, such as pollination, seed dispersal, seedling establishment, plant-plant interactions and also by factors related to habitat heterogeneity, which in turn may produce specific genetic structures at different spatial scales (Loveless & Hamrick 1984; Heywood 1991; Cruse-Sanders & Hamrick 2004). 10 Knowledge of the small-scale spatial genetic structure (*i.e. the spatial distribution of genotypes*) of plant populations can provide useful information to infer the underlying causal processes generating such patterns (Escudero, Iriondo & Torres 2003; Ng, Lee & Koh 2004), including not only key life-history traits such as dispersal and pollination but also local genetic drift, inbreeding and natural selection (Heywood 1991; Ng, Lee & 15 Koh 2004).

Natural selection has been proved to influence genetic variation and its spatial distribution within plant populations at contrasting hierarchical scales (e.g. Jump *et al.* 2008; Parisod *et al.* 2008). Jump and Peñuelas (2005) reviewed studies reporting climate-related changes in genetic diversity within populations of several plant species. 20 In these studies, evolutionary changes occurred as a response to the selective forces imposed by drought, warming, changes in photoperiod and temperature, and candidate loci for selection were identified using proper population genetic tools (e.g. Bonin *et al.* 2006). Far less attention has been paid to the role of biotic interactions in shaping the genetic structure of a particular plant species. It is known that the genetic variation 25 within a single plant population can influence the structure of the dependent community and community-level processes (Whitham *et al.* 2006; Tétard-Jones *et al.* 2007). The

field of community genetics (i.e. the study of the genetic interactions among species and their abiotic environment; Whitham *et al.* 2003) arises from the idea that the genetic structure of dominant species can affect associated species within the community (Whitham *et al.* 2006; Whitlock *et al.* 2007). So far, the study of the effects of genetic  
5 variation on the ecology of coexisting species has mainly focused on defence mechanisms and plant-animal interactions (e. g. Crutsinger, Cador & Sanders 2009), while plant-plant interactions have seldom been addressed (but see Fridley, Grime & Bilton 2007 or Johnson *et al.* 2008).

Effects of the genetic structure of a dominant plant on other coexisting species  
10 should be especially important when the species are closely related. Interactions between congeners have received sustained attention since the mid-nineteenth century (see Darwin's naturalization hypothesis, Darwin 1859; Tansley 1917; Jarvinen 1982), and there is ample experimental evidence of competition between congeneric species (e.g. Rice & Nagy 2000; Brown, Mitchell & Graham 2002; Matesanz, Valladares &  
15 Escudero 2009). If the coexisting species are close congeners, they may share suites of functional traits and are therefore likely to compete for the same resources and in a similar way. We thus argue that the interactions between congeners are stronger and thus more easily detectable than those among non-congeneric species.

In this study we combined spatial statistics and molecular techniques to  
20 understand how two closely related congeneric thymes (*Thymus vulgaris*, a widely distributed species, and *T. loscosii*, a rare narrow endemic) interact at the local scale. The two species belong to the same section of the genus and share functional traits related to pollination, resource use, sexual polymorphism and dispersal structures (Morales 1986). Because of resource-use and functional trait similarities between the  
25 study species, we hypothesize that the spatial patterns of both species when they co-occur is not random but dissociated due to competition, and consequently the spatial

genetic structure of the study species may be shaped by the interaction among them. Specifically, we expect that both the spatial patterns and genetic structure of the narrowly distributed species are dependent on the spatial structure of its more widely distributed congener. To test these hypotheses, we first quantified the spatial patterns and the association of the two species when they coexist and secondly, we used inter-simple sequence repeat (ISSR) molecular markers to determine whether coexistence influences the genetic variation of either of the two species and/or the spatial genetic structure of the populations of each species.

## 10 **Materials and Methods**

### STUDY SITE, SPECIES AND SAMPLING DESIGN

The study was carried out in a gypsum dwarf shrubland in Viana, Spain (42° 30' 45''N; 2°22'18''W; 430 m a.s.l.). Climate is continental Mediterranean, with a broad range of temperatures both over the year and during the day, and pronounced summer droughts.

15 See Matesanz, Escudero & Valladares 2009 for detailed long-term and study year's climatic data and Table S1 for soil water content and soil properties at the study locality.

Plant cover is generally low (around 50-60%). Together with the studied species (*Thymus vulgaris* L. and *Thymus loscosii* Willk.) other common species in these plant communities are *Lepidium subulatum* L. (Cruciferae), *Helianthemum squamatum* (L.) Dum. Cours (Cistaceae), *Rosmarinus officinalis* L. (Labiatae) and *Santolina chamaecyparissus* L. (Asteraceae). *T. loscosii* and *T. vulgaris* (Labiatae) are Mediterranean chamaephytes of contrasting distributions. Both species belong to the same section within the genus *Thymus* (section *Thymus*; Morales 1986; 2002) and often co-occur in the wild. They share traits regarding pollination and sexual polymorphism. See Table 1 for a detailed description of both species.

In May 2002, a 10 x 10 m plot was established in the field in a site where the two species coexist. The plot was haphazardly established in a representative and relatively flat area (slope less than 10%) in which these two species were dominant and at similar high densities. Due to the rarity of *T. loscosii*, which occurs only on a much  
5 reduced area, this selection was almost obligate (García 2007; Matesanz *et al.* 2009). This plot size also guaranteed the inclusion of a very high number of individuals of the two study species. Preliminary estimates in these types of semiarid plant communities of the number of total perennial individuals showed values ranging between 30 and 70 (e.g. Caballero *et al.* 2003). The plot was divided in 100-1 m<sup>2</sup> quadrats. The selected  
10 quadrat size is appropriate to quantify the spatial patterns of the study species in these communities, as it includes several individuals of each study species (Legendre & Legendre 1998). Cartesian (x, y) coordinates were assigned to the centroid of each quadrat. In each quadrat, the **number of individuals (hereafter abundance)** and the cover of *T. vulgaris* and *T. loscosii* were recorded. In order to partial out the effects of the  
15 abiotic environment and other community-level properties in the spatial patterns of these congeners, we recorded soil water content in each quadrat with a soil moisture sensor (ThetaProbe, Delta-T Devices, Cambridge, United Kingdom). Soil water content has been proven to be one of the most critical factors in the performance of plants in semiarid conditions as the ones in our study (see e.g. Escudero *et al.* 1999, 2000,  
20 Matesanz, Valladares & Escudero 2008). Also, we recorded total cover, species richness and cover of each present perennial species in each of the 100 quadrats. Shannon Index of diversity was calculated for each quadrat. Finally, we collected leaf tissue from one individual of each study species per quadrat for the molecular analyses and we kept them frozen at -80°C. Samples were collected as close to the centroid of the quadrat as  
25 possible, so that the distance between sampled plants was kept constant. We collected

100 georeferenced leaf samples of *T. vulgaris* and 72 georeferenced leaf samples of *T. loscosii*, as this species was not present in all the quadrats.

#### ANALYSES OF FINE-SCALE SPATIAL PATTERNS

5 Fine-scale spatial patterning of the cover and abundance of each species was analyzed using the spatial analysis by distance indices methodology (SADIE; Perry 1998). SADIE is based on the distance to regularity ( $D$ ), which measures the total distance in the space that the variable under study would need to move to achieve an arrangement where all the sampling points in a quadrat have the same value. Division of  $D$  by the  
10 average value obtained from permutations where the values of the variable under study are randomly arranged gives an index of aggregation,  $I_a$ , which quantifies the spatial pattern. A clumped spatial pattern is indicated by  $I_a > 1$ , a random pattern has a  $I_a$  close to 1, and a regular pattern has a  $I_a < 1$ . The higher the  $I_a$ , the more spatially clumped the variable under study. It also provides the local index of clustering ( $v$ ), which measures  
15 the degree of clustering of the data into patches (areas with above-average counts of the study variable) and gaps (areas with below-average counts). The index of clustering is determined using data obtained from the redistribution of points described above (see Perry *et al.* 1999 for a full description of the method). In addition to determining the spatial distribution of a variable, it is possible to analyze spatial associations between  
20 variables. Two variables may be spatially positively associated, negatively dissociated, or occur at random with respect to one another. Local spatial association can be measured using an index ( $\chi_i$ ) based on the similarity between the clustering indices of the two variables measured locally at the  $i$ th sample quadrat (Winder *et al.* 2001). If the values of  $v$  (local cluster index) for the first species are denoted  $v_1$ , with mean  $q_1$  and  
25 those of the second are denoted  $v_2$ , with mean  $q_2$ , a measure of local spatial association for a sampling unit  $i$  ( $\chi_i$ ) is given by:



$$\chi_i = \frac{n(v_{i1} - q_1)(v_{i2} - q_2)}{\sqrt{\sum_i (v_{i1} - q_1)^2 (v_{i2} - q_2)^2}}$$

where n is the number of quadrats (100 in our study; Winder *et al.* 2001). The overall spatial association, X, is the mean of these local values,  $X = \sum_i \chi_i / n$ ; Also, X is the simple correlation coefficient between the clustering indices of each variable. Positive and negative values of X denote positive and negative overall spatial association between species, respectively.

We quantified the spatial patterns of each study species, and the spatial association between them. Significance in each case was assessed using a randomization test with the maximum number of randomizations allowed by the program (5967 and 10000 for spatial pattern and spatial association, respectively) applying sequential Bonferroni correction to account for multiple comparisons and Dutilleul (1993) correction for the degree of autocorrelation. An experimental omnidirectional semivariogram (the basic function that describes the spatial dependence of a given variable taking into consideration all the directions in the space, Legendre & Legendre 1998) was built for the local index of clustering ( $v$ ) of each variable (total cover, *T. vulgaris* cover, *T. loscosii* cover, **abundance** of *T. vulgaris* and **abundance** of *T. loscosii*) using Gstat (Pebesma & Wesseling 1998). The fitted semivariograms were used to plot the contour maps in Surfer 8 (Golden Software Inc. Colorado, USA).

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## MOLECULAR ANALYSES

We used ISSR markers (Inter-Simple Sequence Repeat) for the genetic characterization of the sampled individuals (Zietkiewicz, Rafalski & Labuda 1994). The principle of ISSRs is that primer sites are dispersed throughout the genome so that there is a high probability of the primer binding to two sites located on opposing DNA strands within an amplifiable distance of one another. Thus, single-primer amplifications often result

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in a high degree of polymorphic bands and highly reproducible band patterns (Wolfe, Xiang & Kephart 1998).

DNA was extracted from 50 mg of frozen leaf tissue. Leaf tissue was manually disrupted in liquid nitrogen and DNA extracted using the DNeasy Plant Mini kit provided by Qiagen (The Netherlands). The UBC Microsatellite Primer set (University of British Columbia, Canada) was used for primer selection. A minimum of 20 leaf samples per species were used with each primer (from a total of 30 primers screened), and 7 primers were finally selected for DNA amplification (Table 1). Selection criteria were based on the number of amplified and polymorphic bands and the reproducibility of the band patterns. Selected primers were those that rendered polymorphic and repeatable bands in 5 consecutive tests. The PCR reactions consisted of 10.5 µl distilled water, 2 µl buffer (Tris HCL 75 mM, MgCl<sub>2</sub> 2 mM, KCl 50 mM, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 20 mM), 0.4 µl formamide, 0.4 µl DNTP, 2 µl primer 5 µM (Metabion, Germany), 0.7 µl DNA polymerase (Biotools, B & M Labs, S.A, Madrid, Spain) and 4 µl sample DNA. An Eppendorf Mastercycler (Eppendorf, Westbury, NY) was used with the following settings: 60 s at 94°C, 35 cycles of 30 s at 94°C, 45 s at 52°C and 120 s at 72°C, and a final cycle of 5 min at 72°C. After the PCR, 7 µl of PCR products were mixed with 2 µl of bromophenol blue marker and resolved electrophoretically on 2.5% agarose gels (Agarose D-1 Low EO, Pronadisa, Conda Lab., Madrid, Spain). The gels were run at constant voltage (120 V) in 1x Tris-acetate-EDTA until the marker dye migrated 12 cm (≈2 h). The gels were then visualized by staining with ethidium bromide, and photographed under ultraviolet light. Molecular weights were estimated using a 50-2000 bp DNA ladder (Biotools, B & M Labs, S.A, Madrid, Spain). Since ISSR markers are dominant, we assumed that each band represented the phenotype at a single biallelic locus. Amplification products were then treated as phenotypes, where each band represented a character with present/absent states (1 or 0, respectively). Two observers

scored each ISSR band of each sample. Only bands of size lower than 1200 bp were considered, to minimize size homoplasy of bands (Vekemans *et al.* 2002).

To characterize ISSR variation, we first calculated the numbers of shared and unique bands and the percentage of polymorphism. Also, we constructed a matrix of ISSR phenotypes for each species, where individuals were represented by vectors of ones and zeroes across all primers. For diversity measurements, Shannon Index was computed to provide an estimate of the degree of variation within the population of each species, using POPGENE (Yeh & Boyle 1997) and the formula corrected for ISSR data:  $S = -\sum p_i \log_2 p_i$ , where  $p_i$  is the frequency of presence of each ISSR band. In addition, the proportion of distinguishable genets (Ellstrand & Roose 1987) was quantified for *T. loscosii* as  $G/N$ , where  $G$  is the number of genets and  $N$  is the total number of ramets sampled.

#### ANALYSES OF GENETIC STRUCTURE

To characterize the spatial genetic structure of each species, we built a genetic distance matrix for each species using the phenotype matrix, which is built computing the Jaccard's distance index with the bands coded as 1's and 0's for each pair of individuals (Zietkiewicz, Rafalski & Labuda 1994). Likewise, a geographic distance matrix was constructed using the quadrat coordinates as a surrogate for the genotyped individual distances. Individual distance matrices were also built for total cover (computing total cover distances between quadrats using the Euclidean distance among them), cover of each species (with the Euclidean distance), **abundance** of each species (Euclidean distance), soil water content (Euclidean distance), Shannon diversity Index (Euclidean distance) and community composition (Bray-Curtis distance). Distances were then calculated as  $(1-\text{similarity})^{0.5}$ . See Legendre & Legendre (1998) for calculations and suitability of different distance indexes. As *T. loscosii* was not present in all the sample

quadrats, different matrices were calculated for each variable using only those sample quadrats where *T. loscosii* was present.

The relationship between the genetic distance matrix of each species and the distance matrices of the measured variables (geographic distance, total cover, abundance, etc.) was estimated using Mantel tests (Legendre & Legendre 1998). Mantel coefficients obtained with this test can be interpreted as a parametric Pearson correlation among similarity indices. Statistical significance of Mantel tests were tested using a randomization approach with 999 permutations. When the correlation between two matrices could be influenced by another matrix, a Partial Mantel test was performed using the third matrix as a covariable (see Results section). This technique is similar to a partial correlation, being able to detect the correlation between two matrices of interest when the effect of a third matrix is partialled out (Legendre & Legendre 1998).

When a relationship between two matrices was detected, the shape of the genetic structure across the corresponding space was evaluated using a Mantel correlogram. This technique tests for spatial autocorrelation overall and at different distance classes from a multivariate perspective, computing a correlogram for multivariate data using the Mantel statistic ( $r_M$ ) and a permutation test for significance (Escudero, Iriando & Torres 2003; Legendre & Legendre 1998). Each distance class includes all the pairs of points that are located a specific distance from each other. A single correlation index is then calculated for each distance class. The distance intervals (size of distance class) and the number of distance classes were calculated using Sturge's rule (Legendre & Legendre 1998), starting at the minimum distance among the centroid of neighbor sample quadrats (1 m). In order to test the significance of each distance class, 999 permutations were performed. The progressive Bonferroni correction was used to account for multiple testing (Legendre & Legendre 1998). To our knowledge, Mantel correlograms have been used only for geographic distances between sampled plots. In this study, we

use a novel approach in which the geographic spatial metrics are extended to other Euclidean distances based on other predictors such as the distance in the abundance of conspecific congeners.

Distance matrices, Mantel test and Mantel correlograms were performed in the R statistical environment (R Development Core Team 2009), using packages “vegan” (Oksanen *et al.* 2009), “ade4” (Dray & Dufour 2007), and “mpmcorrelogram” (code available in Appendix 1).

## Results

### 10 DESCRIPTION OF THE PLANT COMMUNITY

Average perennial cover in the plot was  $62.1 \pm 1.3$  %. Fifteen perennial species were present in the study plot (total number of individuals of perennial species present in the  $100 \text{ m}^2$  plot was 3520; average Shannon diversity Index was  $1.82 \pm 0.04$ ). The two study species were the most abundant, accounting for ~40% of the total plant cover in the plot (12% and 30% cover for *Thymus loscosii* and *T. vulgaris*, respectively). A total of 476 individuals/ramets of *T. loscosii* were recorded in the study plot (average abundance was  $4.8 \pm 0.6$  individuals/ $\text{m}^2$ , range 0-30); a total of 2778 *T. vulgaris* plants were present in the plot (average abundance was  $27.8 \pm 1.0$ , range 1-57).

### 20 SPATIAL PATTERNS AND SPATIAL ASSOCIATION

The spatial pattern of the cover of *T. vulgaris* showed an overall **dissociation** with that of *T. loscosii* as shown by the negative significant index of association ( $X = -0.32$ ;  $P < 0.01$ ). Contour maps of local association showed areas of local dissociation between the species (Fig. 1 top left). A significant **dissociation** was also found between the spatial patterns of the cover of *T. vulgaris* and the **abundance** of *T. loscosii* ( $X = -0.298$ ;  $P <$

0.01; Fig. 1, bottom left), and between the **abundance** of each species ( $X = -0.270$ ;  $P < 0.01$ ; Fig. 1, bottom right).

All the study variables measured in each quadrat showed a significantly clumped pattern, as shown by the significant values of  $I_a$  (Table S2). Contour maps of the local index of aggregation for the study species showed a patchy distribution of the cover of each species in high density and gap areas (data not shown).

### SPATIAL GENETIC STRUCTURE OF THE STUDY SPECIES

The genetic distance matrix of *T. vulgaris* was not significantly correlated with the genetic distance matrix of *T. loscosii* ( $r_M = 0.04$ ;  $P = 0.325$ ), i.e., the spatial pattern of the genotypes of the two species was not coupled. Likewise, the genetic distance matrix of *T. vulgaris* was not significantly correlated with the corresponding matrix of geographic distances (Table 2). However, we found a significant correlation between the genetic matrix of *T. loscosii* and the matrix of geographic distances (Table 2). We also found a significant correlation between the genetic matrix of *T. loscosii* and the matrix of total plant cover distances. In addition, we found a positive relationship between the matrix of genetic distances of *T. vulgaris* and the distances in the **abundance** of *T. vulgaris* between quadrats, and the same relationship was found for *T. loscosii* (Table 2). We did not find a correlation of soil water content, community composition and diversity index with the genetic matrix of either species (Table 2).

In order to assure that the observed relationships were not spurious, a Partial Mantel test was then carried out between the genetic matrix of *T. loscosii* and the matrix of geographic distances, controlling for the effect of total cover of the community and **for the abundance** of *T. vulgaris* (Table 2). The test rendered very similar results in both cases, revealing the independent contribution of space to the genetic structure of *T. loscosii*. Likewise, the relationship between the distances in the **abundance** of *T.*

*vulgaris* and the genetic matrix of *T. loscosii* remained significant after correcting for the effect of total cover and geographic distances (Table 2). On the contrary, the relationship between total cover and the genetic distance matrix of *T. loscosii* was not significant after correcting for the effect of geographic distance or the **abundance** of *T. vulgaris* (Table 2).

To explore the spatial scale and the shape of these genetic structures, we computed Partial Mantel correlograms for the spatial genetic structure of each species. *T. vulgaris* did not show any significant autocorrelation for any distance class (Figure 2, top left). Conversely, the correlogram for *T. loscosii* was globally significant ( $P < 0.05$ ), and showed significant and positive autocorrelation in the first two distance classes (up to  $\approx 3$  m). For distances between 5 and 9 m, significant negative values of autocorrelation were also found (Figure 2, top right). See Table S2 for detailed results of these Mantel correlograms.

The Partial Mantel correlogram of the genetic structure of *T. vulgaris* with the distance in the **abundance** of *T. vulgaris* showed no significant correlations for any distance classes (distances in terms of the number of individuals), despite the overall significant relationship found with the Partial Mantel test (Table 2; Fig. 2, bottom left). In contrast, we found significant positive autocorrelation for the first two distance classes in the case of *T. loscosii*. This means that *T. loscosii* plants located in sample quadrats containing a similar **abundance** of *T. vulgaris* are genetically more similar than what would be expected by chance, and those situated in sample quadrats containing very different numbers of individuals of *T. vulgaris* are genetically more dissimilar (see Fig. S1 for a graphical example).

The total number of scored bands in the 100 studied individuals of *T. vulgaris* was 54, of which 25 were polymorphic (46.3%). A hundred different genotypes were found. The number of bands amplified per primer was in the range 10–13, (CT)<sub>8</sub>RG being the most polymorphic primer (Table 3). The percentage of polymorphic bands ranged from 27.5% ((AC)<sub>8</sub>YG) to 70% ((CT)<sub>8</sub>RG). Fragment size varied from 290 to 1100 bp. Shannon Index of genetic diversity was  $0.603 \pm 0.102$ . The total number of scored bands in the 72 *T. loscosii* studied individuals was 48, of which 18 bands were polymorphic (37.5%). The number of bands amplified per primer was in the range 7–11, (AC)<sub>8</sub>CYT being the most polymorphic primer (Table 3). The percentage of polymorphic bands ranged from 30% (HVH(TG)<sub>7</sub>) to 54.5% ((AC)<sub>8</sub>CYT). Fragment size varied from 390 to 870 bp. Shannon Index was  $0.550 \pm 0.144$ . A total of 57 different genotypes were distinguished, so the percentage of distinguishable genets was 79%. We found three pairs of individuals and an additional group of seven individuals with the same genotype in *T. loscosii* that were considered four distinct clones. Distance among these clones varied from 1 to 6 m.

## Discussion

Our study showed complex spatial patterns and genetic structure of the two study species. We found spatial dissociation between the spatial patterns of both species, and most interestingly, we found a pattern in the arrangement of *Thymus loscosii* genotypes in relation to the local abundance of *Thymus vulgaris*, suggesting that interactions between the two species at the local scale might shape the spatial genetic structure of *T. loscosii*.

It is well known that unveiling spatial structures of coexisting species is key to determining past processes shaping plant-plant interactions (McIntire & Fajardo 2009). Our results showed significant spatial dissociation between the two thyme species. This



pattern was found both for the cover and the abundance of the two species, and concurs with previous studies reporting competition between close relatives (Tansley 1917; Jarvinen 1982; Rice & Nagy 2000). Interestingly, in a previous study with these two species carried out in the same study site (Matesanz *et al.* 2009), we found changes in the spatial pattern of *T. loscosii* from random when *T. vulgaris* was not present to clumped when the species coexisted, suggesting competition between the two species. Although the mechanisms underlying these patterns cannot be elucidated exclusively with the study of spatial patterns, many studies have related spatial association of two species to facilitation, and spatial dissociation to competition (e.g. Miriti 2007).

Accordingly, it could be expected that the spatial genetic structure of the two species is shaped by competition between them. Interestingly, we found a pattern in the arrangement of *T. loscosii* genotypes in relation to the local abundance of *T. vulgaris* (Table 2), as shown by the Partial Mantel correlogram (Fig. 2). **Individuals of *T. loscosii* in any two quadrats were genetically more similar where the abundance of *T. vulgaris* was similar (short distance classes in the correlogram).** It is worth highlighting that this pattern is still present when the Mantel test is corrected for the effect of geographic distance (as shown by the partial Mantel tests in Table 2), i.e. **the observed pattern holds regardless of spatial distance between the quadrats. An explanation for this finding would be** that the genetic structure of *T. loscosii* is associated with changes in the habitat, in this case directly or indirectly produced by the presence of a congeneric species. As the **abundance** of *T. vulgaris* varies, it may create different environmental conditions for the growth of *T. loscosii* and hence select for different genotypes – **genotypes with different competitive ability**– of the latter species. We also found a weaker density-dependent effect in the case of *T. vulgaris*. Local abundance of conspecific individuals was correlated with the genetic structure of this species. However, none of the distance classes of the Mantel correlogram were significant. This suggests

that the relationship between the abundance of *T. vulgaris* and the genetic distance of this species is not as intense in the case of *T. vulgaris* as to be structured in a significant correlogram.

Adaptation of co-occurring species to the presence of *T. vulgaris* was reported by Ehlers & Thompson (2004), who found that *Bromus erectus* plants showed an adaptive response to soil modifications mediated by local thyme chemotypes (a chemically distinct entity in a plant that shows differences in the composition of secondary metabolites, Franchomme et al. 1990). Recent studies also show that variation among plant genotypes can influence growth and biomass formation of competing plant species (Johnson et al. 2008; Lankau & Strauss 2007). For example, Fridley, Grime & Bilton (2007) showed that the genetic identity of competing species influenced plant performance and mediated species' responses to environmental variation. However, to our knowledge, our study is the first to report **potential** associations between patterns of molecular marker variation and species interactions.

Molecular markers are invoked to be putatively neutral with regard to natural selection (McKay *et al.* 2001). However, many studies have shown adaptive genetic structure based on neutral markers as a response to environmental heterogeneity (e.g. Li *et al.* 2000; Prentice *et al.* 2000; Vitalis, Dawson & Boursot 2001; Volis *et al.* 2001; Bonin et al. 2006 and references therein). Selection can have an effect over the whole genome due to linkage of certain genomic regions to others that are affected by selection (Storz 2005; Joost et al. 2007). For example, a locus that is neutral will respond to selection if it is in linkage disequilibrium (statistical association among allelic states at different loci) with other loci that are subject to selection (Vitalis, Dawson & Boursot. 2001; Avise 2004), and this might be the case in our study.

Despite the fact that our results **–both the spatial patterns analyses and genetic structure–** are consistent with the hypothesis that the spatial genetic structure of *T.*

*loscosii* could be shaped by the interaction between both species, other processes such as pollination and dispersion patterns, migration or genetic drift cannot be completely discarded. For example, non-random mating mediated by pollination could be contributing to the observed pattern. If pollinators are attracted to patches with similar abundance of *T. vulgaris*, and the same pollinators also visit the neighbouring *T. loscosii* plants (these species share pollinators), pollen flow in *T. loscosii* would happen more often among individuals occurring in patches of similar *T. vulgaris* abundance. This could increase the genetic similarity of *T. loscosii* plants growing in similar-abundance patches. In this scenario, it would also be reasonable to expect non-random mating to affect *T. vulgaris* and therefore observe higher genetic similarity of the plants of *T. vulgaris* occurring in patches of similar abundance. However, the fact that *T. vulgaris* shows no spatial genetic structure suggests that non-random mating would unlikely be the only mechanism responsible for the observed patterns.

An additional alternative explanation could be that a different unmeasured abiotic factor is regulating both the abundance of *T. vulgaris* and the fine-scale genetic structure of *T. loscosii*, and hence our findings could be the result of a spurious correlation. If an unmeasured abiotic factor was regulating both the abundance of *T. vulgaris* and the spatial genetic structure of *T. loscosii*, we would expect to observe correlations between the abundance of *T. vulgaris* and other response variables as well (e.g. total cover, abundance of *T. loscosii*, etc). However, the abundance of *T. vulgaris* was only correlated with the genetic matrix of *T. loscosii* and not to any of the measured predictors such as water or diversity (which would likely be correlated with almost any unmeasured predictor), suggesting that the observed correlation is not spurious and regulated by a third factor. Notwithstanding, to accurately interpret the observed genetic structure as driven by selection, appropriate tests of individual fitness of *T. loscosii*

genotypes under different abundances of *T. vulgaris* should be experimentally tested, including if possible other areas within the study locality as well as other sites.

We did not find any significant spatial genetic structure of the widespread *T. vulgaris*. While both species showed significant clumped distributions in terms of cover and abundance, only *T. loscosii* showed a significant spatial genetic structure. Non-random genetic patterns can exist without a non-random distribution of individuals, and conversely, a plant population may show a non-random spatial distribution without any accompanying genetic structure (Loveless & Hamrick 1984; Heywood 1991). The latter is the case of *T. vulgaris*, which showed high values of the index of aggregation for most variables ( $I_a$  of cover and **abundance**; see Table S2) but no spatial genetic structure, at least at the fine-scale of our study (i.e. closer plants were not genetically more similar than plants located at greater distances). Spatial aggregation in this species may have arisen from the heterogeneous distribution of favourable microsites for germination and establishment typical of semiarid environments (Aguilar & Sala, 1999), from dispersal patterns (Loveless & Hamrick 1984; Heywood 1991; Stoll & Prati 2001) or from mortality patterns over the course of ontogeny (De la Cruz *et al.* 2009). Dispersal is atelechorous because seeds lack an active dispersal mechanism. Seeds mainly disperse by gravity, having only a mucilaginous coat that helps to anchor dispersed seeds in the close vicinity of their mother plants (Morales 1986). This could have led to a high degree of genetic structure resulting from clusters of related seedlings growing near the mother plant (Berg & Hamrick 1994; Cruse-Sanders & Hamrick 2004). However, the random distribution of genotypes suggests that dispersal distances are either greater than expected (*T. vulgaris* are occasionally dispersed by ants, A. Escudero pers. obs.) or smaller than the minimal sampling distance of our study (1 m).

On the contrary, *T. loscosii* showed significantly clumped genetic patterns (Table 3) and genetic similarity up to 3 m (Fig. 2). This can be due to the clonal growth

in this plant, as has been reported in other clonal plant species showing spatially clumped genetic structures (Stoll & Prati 2001; Eckert 2002). The negative correlation found at greater distances (5-10 m) suggests that individuals located far apart likely originated from different genets. Nevertheless, the degree of clonality is not especially high in this plant, as also shown by García (2007), who found that only half of the *T. loscosii* plants showed clonal ramets in a study performed in the same population as the present study. In fact, the number of putative clones (i.e. samples with the same band pattern) found was low, which suggest a less than expected influence of clonality. The higher proportion of distinguishable genets (0.79) compared to those found in the reviews by Ellstrand & Roose (1987) and Widén, Cronenberg & Widén (1994), which averaged 0.26 and 0.32, respectively, also indicates that sexual reproduction plays an important role in the reproduction of *T. loscosii* in the studied population. Also, the clumped spatial pattern of the abundance of *T. vulgaris* may contribute slightly to the genetic autocorrelation of *T. loscosii* at short distances, as *T. vulgaris*' abundance showed a clumped pattern up to ~2m (compare Mantel test of the genetic matrix of *T. loscosii* and geographic distance and the partial Mantel test when corrected for the abundance of *T. vulgaris*; see also Mantel correlogram of the abundance of *T. vulgaris* in Fig. S2).

Finally, we found moderate levels of genetic variation in both study species. Rare plant species are commonly hypothesized to have little genetic variation compared to widespread congeners because of genetic drift, strong directional selection toward genetic similarity in the few environments where the species is present or inbreeding depression (Molano-Flores *et al.* 1999; Ayres & Ryan 1999; Gitzendanner & Soltis 2000 and references therein). Our results do not support this hypothesis, as we found moderate and similar levels of genetic variation in both study species, as shown by the Shannon Index of genetic diversity and band polymorphism.

Collectively, our results reveal spatially complex genetic structures of both congeners at small spatial scales. While both species showed similar levels of genetic variation, they differed in their spatial genetic structure, which may be due to the contrasting reproduction strategies of the two species. Likewise, the negative association among the spatial patterns of the two species and the genetic structure found for *T. loscosii* in relation to the abundance of *T. vulgaris* suggest that competition between the two species may account for the presence of adapted ecotypes of *T. loscosii* to the abundance of a competing congeneric species. This suggests that the presence and abundance of close congeners can influence the genetic spatial structure of plant species at fine scales.

## Acknowledgments

The authors would like to thank B. Pías and colleagues from URJC, especially L. Giménez-Benavides and A. Sánchez for their inestimable help during the lab work. T. Culley, R. Piñeiro and R. Agudo provided their expertise and help during ISSR data analyses. JM Iriondo, J. Herman and P. Mason provided useful comments in the manuscript. Funding was provided by the Spanish Ministry for Innovation and Science with the grants Consolider Montes (CSD2008\_00040) and VULGLO (CGL2010-22180-C03-03), and by the Community of Madrid grant REMEDINAL 2 (CM S2009/AMB-1783).

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**Table 1.** Comparative description of the two study species. Extracted from Morales (1986, 2002).

<b>Description</b>	<i>T. loscosii</i>	<i>T. vulgaris</i>
<b>Life cycle</b>	Perennial	Perennial
<b>Raunkiaer's life form</b>	Chamaephyte	Chamaephyte
<b>Reproduction mode</b>	Sexual and clonal (stolons)	Sexual
<b>Sexual polymorphism</b>	Gynodioecious	Gynodioecious
<b>Growth habit</b>	Creeping	Erect
<b>Pollination</b>	Entomophilous ( <i>Apis mellifera</i> and some some Bombylidae)	Entomophilous ( <i>Apis mellifera</i> and some some Bombylidae)
<b>Flowering peak</b>	June	April-June
<b>Distribution</b>	Rare species; Narrow endemic to the Ebro river valley in north-eastern Spain; 100-1300m a.s.l.	Widespread species, endemic to the Mediterranean Basin; 200-2200 a.s.l
<b>Conservation status</b>	Of special interest (BOE 1990)	NA
<b>Type of climate</b>	Continental Mediterranean (400-600 mm annual rainfall)	Mediterranean. Broader variation of environmental conditions
<b>Type of soil</b>	Gypsum, loam, limestone	Broad variation of soils
<b>Plant height</b>	~15cm	~30cm
<b>Chromosome number</b>	2n=54 (tetraploid)	2n=28, 2n=30

**Table 2.** Top: Results of the Mantel test ( $r_M$  and  $p$ -values) among the genetic distances of the individuals of each study species and total cover, cover and abundance (number of individuals) of each of the two species and also community composition.

5 Significance was assessed using 999 randomizations. Bottom: Results of the partial Mantel test ( $r_M$  and  $p$ -values) among the genetic distances of the individuals of each study species. Covariable matrix used is indicated in each case. Significance was assessed using 999 randomizations. Figures in bold are significant after sequential Bonferroni correction.

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		Genetic distance matrix of <i>T. loscosii</i>		Genetic distance matrix of <i>T. vulgaris</i>	
Mantel test		$r_M$	$p$ -value	$r_M$	$p$ -value
Geographic Distance		<b>0.192</b>	<b>&lt;0.001</b>	-0.004	0.914
Soil water content		-0.066	0.882	0.068	0.107
Community composition		-0.024	0.693	-0.060	0.879
Shannon Diversity Index		-0.018	0.661	-0.020	0.649
Total cover		<b>0.104</b>	<b>0.011</b>	0.033	0.231
<i>T. loscosii</i> cover		-0.016	0.623	-0.065	0.267
<i>T. vulgaris</i> cover		0.055	0.163	0.015	0.794
<b>Abundance</b> of <i>T. loscosii</i>		-0.028	0.658	-0.119	0.062
<b>Abundance</b> of <i>T. vulgaris</i>		<b>0.144</b>	<b>0.003</b>	<b>0.127</b>	<b>0.007</b>
Partial Mantel test	Covariable matrix	Genetic distance matrix of <i>T. loscosii</i>			
		$r_M$	$p$ -value		
Geographic Distance	Total cover	<b>0.175</b>	<b>&lt;0.001</b>		
	Nr. Individuals <i>T. vulgaris</i>	<b>0.189</b>	<b>&lt;0.001</b>		
<b>Abundance of <i>T. vulgaris</i></b>	Total cover	<b>0.128</b>	<b>0.003</b>		
	Geographical distance	<b>0.122</b>	<b>0.002</b>		
Total cover	Geographical distance	0.059	ns		
	Nr. Individuals <i>T. vulgaris</i>	0.080	ns		

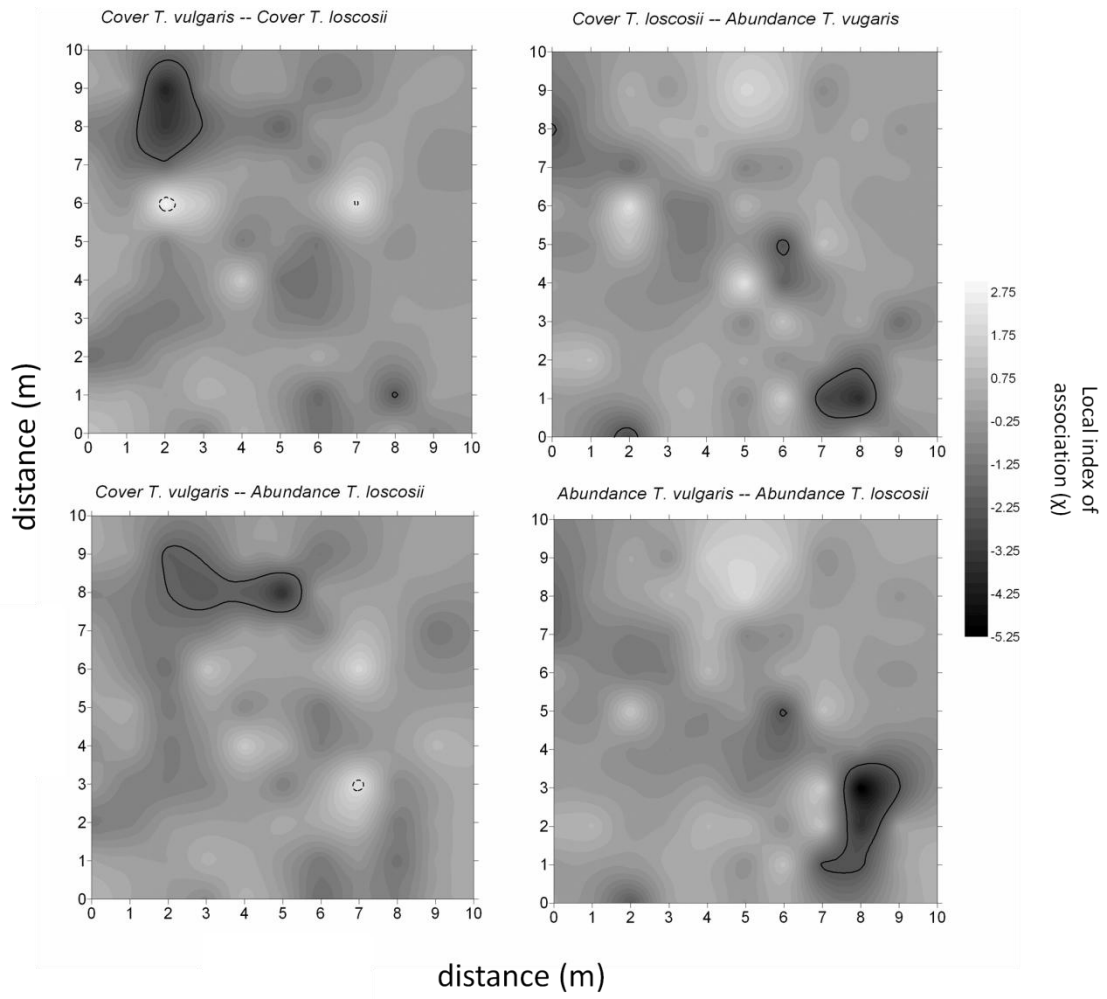
**Table 3.** Primers selected for the molecular analyses of each species, number of amplified bands, number of polymorphic bands and proportion of polymorphism.

	Primer sequence	Amplified bands	Polymorphic bands	Proportion of polymorphism
<i>Thymus vulgaris</i>	(GA) <sub>8</sub> YG	10	4	40 %
	(CT) <sub>8</sub> RC	10	5	50 %
	(CT) <sub>8</sub> RG	10	7	70 %
	(AC) <sub>8</sub> YG	11	3	27.3 %
	HVH(TG) <sub>7</sub>	13	6	46.2 %
	Total bands amplified	54	25	46.3 %
<i>Thymus loscosii</i>	(GA) <sub>8</sub> C	10	3	30 %
	(CT) <sub>8</sub> RC	10	4	40 %
	(AC) <sub>8</sub> CYT	11	6	54.5 %
	(AC) <sub>8</sub> YG	7	2	28.6 %
	HVH(TG) <sub>7</sub>	10	3	30 %
	Total bands amplified	48	18	37.5 %

## Figure legends

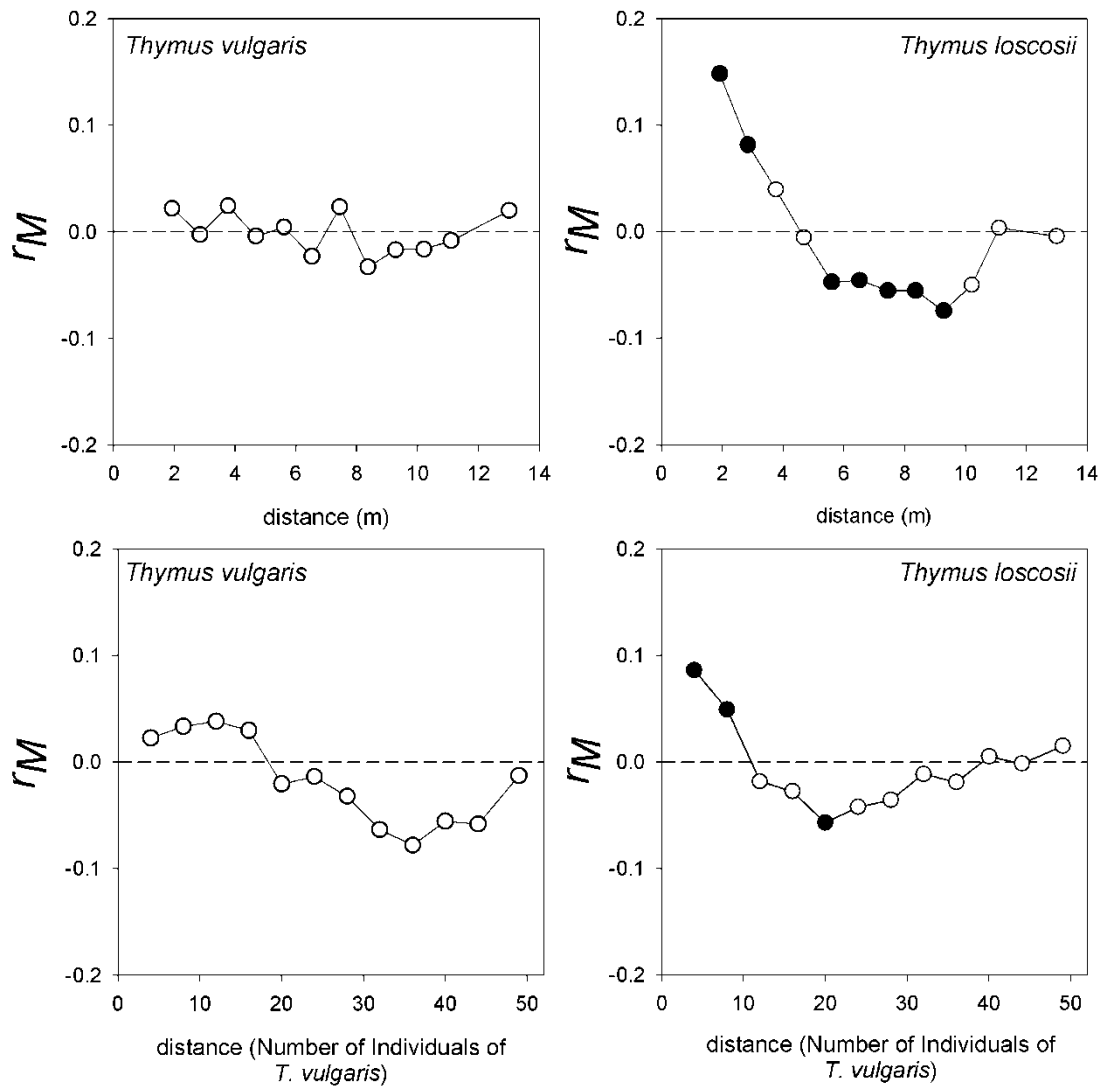
- Figure 1.** Contour maps of local association ( $\chi$ ) between *Thymus vulgaris* and *Thymus loscosii*. Color scale shows the dispersion of the local association index. Dark areas in the maps show local dissociation and light areas show local association. Significant positive or negative association areas ( $p < 0.05$ ) are included within dashed or solid lines, respectively. In all cases, overall spatial association ( $X$ ) was significantly negative. Data was taken at a scale of 1 m<sup>2</sup> quadrats. See text for details.
- 5
- Figure 2.** Top: Mantel correlogram for the genetic spatial structure of *Thymus vulgaris* (left) and *Thymus loscosii* (right). Bottom: Partial Mantel correlogram for the genetic structure of each *Thymus vulgaris* (left) and *Thymus loscosii* (right) in relation to the abundance (number of individuals) of *Thymus vulgaris*. Geographical distance matrix was used as the covariable matrix. Closed circles show significant correlation after sequential Bonferroni correction. See text for details.
- 10
- 15

**Fig. 1**



5

**Fig. 2**



5



## Appendix

**Table S1.** Soil water content and soil properties values in the study locality (N = 9 samples were collected). The soil samples were collected in the surrounding of the study plot. See Matesanz *et al.* 2009 for details on the collection and analyses of the soil nutrients.

	<b>Soil Water content (%)</b>	<b>pH</b>	<b>Organic Matter (%)</b>	<b>Total Carbon (%)</b>
Viana	11.9091 ± 0.2132	7.8387 ± 0.0192	3.5587 ± 0.1286	2.0675 ± 0.0759

**Table S2.** Spatial pattern (Index of aggregation,  $I_a$ ) of the study variables. P-values derived from randomization tests statistics (5967 randomizations) are shown in brackets. See text for details on calculations of the index.

5

<b>Variable</b>	<b><math>I_a</math></b>	<b>Pattern</b>
<i>T. vulgaris</i> cover	2.082 (<0.001)	Clumped
<i>T. loscosii</i> cover	1.560 (0.003)	Clumped
Abundance of <i>T. vulgaris</i>	2.077 (<0.001)	Clumped
<b>Abundance</b> of <i>T. loscosii</i>	1.741 (<0.001)	Clumped
Total cover	2.332 (<0.001)	Clumped
Soil water content	1.729 (0.0013)	Clumped
Shannon Diversity Index	1.719 (<0.001)	Clumped

**Table S2.** Results of the Mantel correlogram for the genetic spatial structure of *T. loscosii* (top) and *T. vulgaris* (bottom). Only distance classes including at least 50 pairs of points are shown. See text for details on the computation of Mantel correlograms.

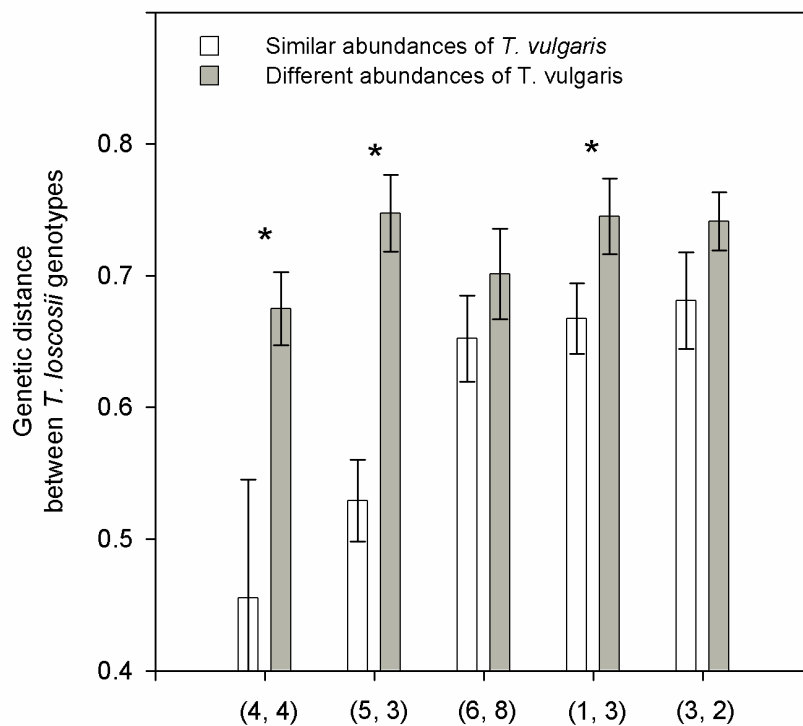
5

Distance class	Midpoint of the class size	Number of pairs	$rM$	$p$ -value	Lower Limit C.I.	Upper limit C.I.
1	1.425	220	<b>0.148</b>	0.001	0.107	0.178
2	2.375	363	<b>0.082</b>	0.001	0.058	0.103
3	3.325	370	0.040	0.072	0.022	0.059
4	4.275	356	-0.005	0.795	-0.022	0.018
5	5.225	359	<b>-0.044</b>	0.018	-0.058	-0.030
6	6.175	306	<b>-0.049</b>	0.011	-0.067	-0.029
7	7.125	237	<b>-0.055</b>	0.013	-0.074	-0.039
8	8.075	199	<b>-0.062</b>	0.015	-0.084	-0.038
9	9.025	104	<b>-0.075</b>	0.019	-0.096	-0.055

Distance class	Midpoint of the class size	Number of pairs	$rM$	$p$ -value	Lower Limit C.I.	Upper Limit C.I.
1	1.425	342	0.010	0.411	-0.003	0.023
2	2.375	576	-0.006	0.688	-0.019	0.008
3	3.325	616	0.008	0.637	-0.006	0.023
4	4.275	626	-0.008	0.617	-0.025	0.008
5	5.225	680	-0.003	0.844	-0.016	0.011
6	6.175	612	-0.025	0.035	-0.038	-0.012
7	7.125	522	0.018	0.228	0.005	0.030
8	8.075	500	-0.006	0.775	-0.021	0.011
9	9.025	312	0.007	0.771	-0.015	0.028
10	9.975	94	0.011	0.519	-0.005	0.027

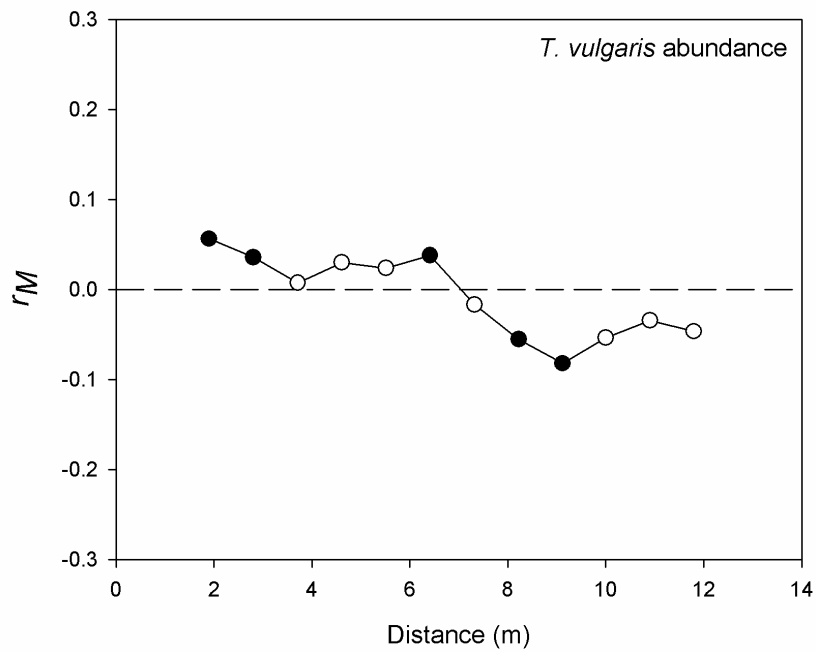
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**Fig S1.** Genetic distances (mean  $\pm$  standard error) between *T. loscosii* plants in quadrats with similar or different abundances (number of individuals) of *T. vulgaris*. Five random 1x1 m quadrats (target quadrats) were selected within the plot (10 x 10 m) for the example (xy coordinates are shown for each selected quadrat). For each target quadrat, the distribution of distances of abundance (i.e. the difference in the number of individuals of each target quadrat with all the quadrats in the plot) was calculated and the 10% and 90% percentile was used to identify quadrats with very similar (lower percentile) and very different (upper percentile) abundances as the target quadrat. Then, the genetic distance between the *T. loscosii* genotype of the target quadrat and the *T. loscosii* genotype of each of the similar and different abundance quadrats was calculated. White and gray bars show the quadrats with similar/different abundance of *T. vulgaris*, respectively. The asterisk shows significant differences in genetic distances between quadrats with similar/different abundance of *T. vulgaris*.



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Fig. S2. Mantel correlogram of the abundance of *T. vulgaris*. Closed circles show significant correlation after sequential Bonferroni correction. See text and Legendre and Legendre (1998) for a detailed description of this technique.



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