

Strong Spatial Genetic Structure Reduces Reproductive Success in the Critically Endangered Plant Genus *Pseudomisopates*

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Abstract

Clonal growth can be a double-edged sword for endangered species, because the short-term insurance against extinction may incur a longer-term hazard of creating small inbred populations with low fecundity. In the present study, we quantify the advantages and disadvantages of clonal growth regarding the fitness of the central Iberian monotypic endangered genus *Pseudomisopates*. Preliminary studies showed that the species is self-incompatible and exhibits extensive clonal growth with plants flowering profusely. However, seeds at many sites seemed to be unviable, and no seedlings have been observed in the field. A fully replicated nested sampling design ($n = 100$) was conducted to explore genetic (using seven SSR loci) and environmental factors potentially affecting seed viability, such as: 1) clonal and genetic diversity, 2) spatial genetic structure, and 3) environmental factors (shrub cover and grazing). Generalized Linear Mixed Models were fitted relating genetic and environmental variables to reproductive variables (seed viability and flower display). Our results indicate that the relatively low genotypic diversity of the population ($PD = 0.23$), as quantified by SSRs, and the strong spatial genetic structure observed are congruent with intense clonal growth. This clonal growth is enhanced by unfavorable environmental conditions, such as canopy closure and grazing. Under these circumstances, both flower display and mate availability decrease, thus hindering sexual reproduction. Indeed, a mixed reproductive system (clonal and sexual) to escape environmental stochasticity is crucial for the survival of *Pseudomisopates*, a species inhabiting a disturbance-prone ecosystem.

Key words: canopy closure, clonality, fitness, herbivory, Mediterranean, *Pseudomisopates rivas-martinezii*, SSR

Clonal growth is very widespread in plants, with about 66.5% of central European flora exhibiting some kind of clonal growth (Klimeš et al. 1997). Clonal growth allows plants to grow and spread horizontally and is more frequent in aquatic plants, plants prone to disturbance, rare and endangered species, alien plants, and at the edge of species geographical ranges (Silvertown 2008). The rate of clonal versus sexual reproduction in natural populations has crucial effects on demography and genetics (Eriksson 1989; Arnaud-Haond et al. 2007), because a certain level of genetic recombination enhances genotypic diversity and the ability to adapt (Balloux et al. 2003). Nevertheless, the advantages of clonality are many and reduce the costs of sexual reproduction, allowing translocation of resources between ramets in heterogeneous environments (Jónsdóttir and Watson 1997; Price and Marshall 1999), demographic benefits such as increased

probability of survival and fecundity (Harper and White 1974), or rapid increase in size and mobility.

But clonal growth could be a double-edged sword for an endangered species because the short-term insurance against extinction might incur a potentially longer-term hazard of creating small inbred populations with low fecundity. In fact, small populations of many clonal species may give the appearance of abundance, though being genetically depauperate (Eckert and Barrett 1993; Sydes and Peakall 1998). In many cases, sexual reproduction can be almost permanently suppressed by environmental conditions such as canopy closure (Kudoh et al. 1999), vegetation succession (Piquot et al. 1996), mowing (Schaal and Leverich 1996), or temperature (Woodward 1990). This situation may lead to lowered genetic diversity or inbreeding, especially in small populations of self-incompatible species, in which

pronounced clonal growth may result in reduced mate availability and plants may not receive enough compatible pollen, thus leading to reduced sexual reproduction or even extinction (Charpentier et al. 2000; Honnay and Bossuyt 2005). However, evidence for this lowered fitness in clonal populations is still scarce (Wolf et al. 2000; Honnay et al. 2006).

Nonetheless, some authors have suggested that the advantages of clonal growth may be larger than the disadvantages imposed by increased geitonogamy and inbreeding depression (Eckert 2000). Indeed for many species living in heterogeneous habitats or depending on disturbance, asexual reproduction might be the best strategy to ensure survival. The clonal nature of many species represents an advantage for colonizing and competing successfully in a range of habitats. Thus, they have become important colonizers of disturbed or manmade habitats, including spoil heaps, and are also dominant in early stages of succession of recently disturbed habitats or early stages of secondary succession (Prach and Pyšek 1994). The evolutionary maintenance of clonality in self-incompatible species will depend on the relative benefit of producing higher-quality offspring promoted by outbreeding versus the reduction in offspring number due to lowered mate availability (Vallejo-Marín and Uyenoyama 2004) and the reproductive compensation over multiple breeding seasons (Goodwillie 1999; Larson and Barrett 2000).

The present study focuses on the advantages and disadvantages of clonal growth in the Iberian endangered species *Pseudomisopates rivas-martinezii* (Plantaginaceae). Although plants flower profusely in this species, most seeds seem to be unviable, as shown by preliminary germination tests and the apparent absence of seedlings at sites monitored over a period of 5 years (Martínez Rodríguez et al. 2004; Amat et al. 2011). The hypothesis that the low levels of sexual reproduction observed on this self-incompatible species may be a consequence of the extensive clonal growth and certain environmental factors was tested. Our aims were to: 1) determine the level of sexual versus asexual reproduction, 2) evaluate the effects of clonality on the genetic structure and the sexual reproduction ability, and 3) determine the environmental factors that enhance clonality. These were assessed by estimating the genotypic and genetic diversity, the genetic structure, as well as the spatial genetic structure (SGS) using SSRs. Furthermore, the relation between genetic and environmental factors (canopy closure and grazing) and fitness variables (flower display and seed viability) were explored.

Materials and Methods

Study Species

Pseudomisopates rivas-martinezii (Plantaginaceae, tribe Antirrhineae) is an endangered species endemic to the Gredos region in central Spain. It has been classified as Critically Endangered following the IUCN criteria in the Red List of Spanish Vascular Flora (Martínez Rodríguez et al. 2004). It is one of the 19 monotypic genera endemic to Spain (Nieto

Feliner 1999), of which five are critically endangered. The species occurs in the *Cytisus oromediterraneus* shrublands from 1400 to 1990 m above sea level. Populations preferably occupy clearings within this habitat, where plants show higher vigor than in the dense shrubland. Two main population areas have been found separated by 20 km, one in Gredos Mountain Range and the other in La Serrota Mountain Range, comprising seven and three populations, respectively (Vargas and García 2008). Plants are long-lived and ramets typically resprout each year (Martínez Rodríguez et al. 2004). Excavation of plants revealed that plants multiplied vegetatively by an underground stolon network, with connections between ramets that can span over 1 m. The species is predominantly self-incompatible and insect-pollinated (Amat et al. 2011). Flowers bloom from July to August and fruiting occurs between August and September. Fruits can contain from 1 to 24 seeds, which are dispersed by barochory.

Study Area and Sampling Design

One population from La Serrota Mountain Range was selected for this study. La Serrota population (*Cepeda de la Mora, Ávila*), in particular, was sampled during the growing season of 2007. The habitat consists of *Cytisus oromediterraneus* shrubland interspersed with granite rocks in a smooth steep hill. Plants grow from 1850 to 1960 m. At higher altitudes plants occur in the shrubland, on the loose soil, and between the granite rocks. At lower altitudes the shrubland has been cleared for pasture and plants grow in pasture grassland. A total number of 21 plant patches, occupying 13 336 m², were found and delimited using a GPS receiver (Garmin Etrex Vista). A Nested Sampling Design was conducted, 10 patches were randomly selected, and 10 sample points were randomly allocated per patch. The nearest plant to these sample points was sampled for leaf material. As a result, 100 ramets (10 from each patch) were analyzed. This sample size was adjusted considering the area of the population, the previous estimate of just 164 genets in this population (Martínez Rodríguez et al. 2004), and the extensive clonal growth of individuals, which expand over metres via underground stolons. About 60 mg of leaf from each ramet was collected and preserved-dried in silica gel. Finally, a dataset of potential drivers on the species reproductive success and habitat suitability was compiled. The diameter, the number of branches, flowers, and fruits were measured from each sampled plant. Each of these plants was taken as the center of a 1m² quadrat in which shrub cover was estimated to the nearest 1%, and ramet density and number of seedlings were counted.

Seed Viability Test

A total number of seven fruits were systematically sampled (when present) from each ramet, and all seeds were counted and tested for viability. As a result, 554 seeds from 51 individuals were analyzed. Seed viability, and hence an estimate of seed germination, was assessed by means of a Tetrazolium test optimized for the species, following the *Tetrazolium Testing Handbook* (Peters 2000). Seeds were imbibed in water overnight at 21 °C, then slightly cut, and incubated in 1% TZ

at 27 °C for 72 h. Seeds were finally bisected and viability was estimated by observing red coloring of living tissues.

DNA Extraction and Microsatellite Protocol

DNA was extracted from leaf material, using the QUIAGEN DNeasy Plant Mini Kit. DNA samples were analyzed with seven polymorphic nuclear microsatellite markers (SSRs) (Table 1) originally genotyped from *Antirrhinum* ESTs (Davies B, University of Leeds, UK, unpublished data) using SPUTNIK (<http://cbib.u-bordeaux2.fr/pise/sputnik.html>) and with primers designed using PRIMER3 (<http://primer3.sourceforge.net/>). A total of 77 markers were tested on *P. rivis-martinezii*, and those amplifying for the species were screened on samples from all known populations, in order to choose the most polymorphic loci. Forward primers were labeled with IRD-800 fluorescent marker. PCR was carried out under the following conditions depending on the primer pair: 5–7 ng of template DNA, 0.2–0.4 µL of each 10 mM primer, 2–2.5 µL of 25 mM MgCl₂, 0.1 µL of 2 mM dNTPs, 0.5 U Taq polymerase. The PCR was run for 4 min for an initial denaturation at 94 °C, followed by 30 cycles of 30 s at 94 °C, 40 s at 55–60 °C (depending on the primer combination), 1.20 min at 72 °C, and a final extension at 72 °C for 10 min to ensure quantitative terminal transferase activity of the Taq polymerase. PCR products were separated on a 6.6% denaturing polyacrylamide gel (40% acrylamide) on a Li-Cor 4200 DNA sequencer. PCR products were sized with a ladder run next to the amplified microsatellites. Fragments amplified by microsatellite primers were scored as present or absent using GeneImagIR version 4.0.

Data Analysis

Clonal Diversity

Clonal diversity was evaluated by calculating the genotypic richness ratio $PD = G-1/N-1$, that is, the proportion of distinguishable genotypes, where G represents the number of multilocus genotypes (MLGs) and N represents the number

of sampled ramets (Dorken and Eckert 2001). This estimator ranges from 0 for monoclonal populations to 1 when all samples belong to distinct genotypes. The p_{sex} statistic (threshold 0.05) was also used to distinguish between identical MLGs that could be the result of distinct events of sexual reproduction (Arnaud-Haond et al. 2007). All calculations were performed using GENCLONE version 2.0 (Arnaud-Haond and Belkhir 2007).

The discriminative power of the polymorphic markers used to differentiate the genotypes (MLGs) present in the sample was explored by: 1) plotting the number of loci versus the number of MLGs detected (Figure S1; see Supplementary Material online), 2) constructing a histogram of genetic distances among MLGs (Figure S2; see Supplementary Material online), and (3) plotting the number of loci versus the number of MLGs detected for each patch (Figure S3; see Supplementary Material online).

Genetic Diversity and Structure

Genetic diversity was measured as mean number of observed alleles per locus (n_a), mean allelic richness per locus (R_s), mean private allelic richness per locus (R_p), mean gene diversity or expected heterozygosity (H_s), observed heterozygosities (H_o) and inbreeding coefficients (F_{IS}). Calculations were performed using FSTAT v.2.9.3 (Goudet 1995). Differences in heterozygosity between shrubland and grassland were explored with a Mann–Whitney U test using STATISTICA 6.0.

Compatible first-degree parentage relationships between accessions were assessed with GIMLET (Valière 2002), although no clear relations were identified. In order to visualize the genetic distances between genotypes, a factorial correspondence analysis (FCA) was carried out using GENETIX 4.05.2 (Belkhir et al. 1996–2004).

Hierarchical structure of genetic variation was examined by an analysis of molecular variance (AMOVA) using ARLEQUIN version 3.1 (Excoffier et al. 2005) to describe the genetic structure and variability among patches in the population. The total genetic variation was partitioned:

Table 1 Data for the seven microsatellites loci: primer sequences, repeat structure, PCR product length and annealing temperature (T_A), and number of alleles amplified per locus for *P. rivis-martinezii*

Locus	Primer sequences (5'–3')	Repeat structure	Product length (bp)	Number of alleles	T_A (°C)
MSAT 35	F- CCTTGGCCITTTCTCTCTCCT R- CCAAGCATCCITTCGGAATA	CTT(9)	236	2	60
MSAT 53	F- TCGACGATGGTGAAGATGAC R- CCCTGAAACGAGAGCGTAAG	TAA(10)	268	2	58
MSAT 61	F- CTCGCCCTCTATCCTCAAA R- TTCGTTGCTGTTGACATGGT	TCT(9)	150	5	60
MSAT 63A	F- CAAGGATTTGTTGGGAAGGA R- ACTAACCCTGGCTTATACGG	AAC(8)	243	4	60
MSAT 63B	F- ACCTCAATTTGGGCACTGAT R- GGTGGAGTTGCTCTTCTTGC	GAA(10)	379	5	60
MSAT 69	F- CACATGTAACCCACGAAAAG R- GGGACCTTCACCAGTACCAA	GT(12)	404	3	58
MSAT 77	F- ACCTCGACGTCAACTTCCAC R- GAGGTTGGGCTTGGGAATAC	GCA(11)	250	2	55

1) between patches and 2) between patches within habitats (pastureland vs. shrubland). The AMOVA was performed at the ramet level, that is, including all samples. The analysis at the genet level considering only one individual per genotype could not be performed due to the high level of clonality, which would lead to an insufficient number of genotypes per patch (one to six, see below), making it unfeasible to partition the variance between patches. Although *F*-statistics and related techniques were developed assuming sexual reproduction in randomly mating populations (McLellan et al. 1997), and even if ramet-level analysis would lead to pseudoreplication, this analysis still provides insights into population structure and is relevant considering the spatial scale of the sampling scheme.

Spatial Structure

First, the spatial distribution of equal genotyped ramets was tested using the aggregation index (*A_i*), and the extension of clones in the population was measured using the clonal sub-range, which is the longest geographic distance between ramets sharing the same MLG (Arnaud-Haond and Belkhir 2007). Second, the SGS between patches was examined using the Mantel test implemented in IBDWS version 3.16 (Jensen et al. 2005). The correlation between the triangular matrix with pairwise geographic distances between patches and the triangular matrix with pairwise *F_{ST}* values between patches was calculated. Finally, spatial autocorrelation analyses were used to confirm the scale dependence of clonal diversity. The average genetic distance or kinship coefficient *F_{ij}* (Loiselle 1995) between pairs of individuals within specific ranges of geographic distance was plotted against 10 spatial distance classes (21, 63, 107, 178, 219, 299, 358, 455, 545, 660 m), among which the number of individual pairs compared was evenly distributed. The *S_p* statistic was calculated at both the ramet and genet level. This ratio is dependent upon the rate of decrease of pairwise kinship coefficients between individuals with the logarithm of the distance in two dimensions and can be used to compare the extent of SGS among populations or species (Vekemans and Hardy 2004). All calculations were performed at the ramet and genet level using SPAGEDI version 1.3.a (Hardy and Vekemans 2002).

Influence of Genetic and Environmental Factors on Reproductive Success

The reproductive variables measured to investigate fitness were first explored: 1) fruit set was estimated as the ratio of fruits to flowers; 2) seed set was estimated as the ratio of seeds to the average number of ovules; and 3) viable seed set was estimated as the ratio of viable seeds to the average number of ovules. Differences in ramet density and grazing frequency between shrubland and grassland quadrats were tested, with ANOVA and Mann–Whitney *U* tests, respectively, using STATISTICA 6.0.

Generalized Linear Models using R (R Development Core Team 2008) were used to explore the relation between unfavorable environmental conditions (canopy closure and grazing) and number of genotypes per patch, first, and the

relation between reproductive variables (flower production, fruit set and seed viability) and clone size, second. Clone size was estimated as patch area by proportion of genotype repetitions.

At a finer scale, Generalized Linear Mixed Models were fitted relating environmental variables to reproductive (flower display) and fitness (seed viability) variables. These analyses followed the hypothesis that flowering would be affected by environmental factors, whereas seed viability would be affected by genetic factors. Fixed effects quantify the overall effects of canopy closure, plant size, and grazing in the first model, and minimum distance to a different genotype and flower display in the second model; random effects quantify the variation across patches of the fixed-effect parameters. The response variables were count data following a Poisson distribution for number of flowers, and Negative Binomial for mean viable seeds. Analyses were performed using the packages “lme4” for the response variable number of flowers and “glmm.admb” version 11 (Fournier et al. 2012) for the response variable mean number of viable seeds per fruit, both packages written for R (R Development Core Team 2008). Model selection was accounted for by first exploring independent variables separately and then considering the most ecologically informative combinations in models including no more than three variables. The best fitting models were finally selected according to the Akaike’s information criterion (AIC) (Table S3; see Supplementary Material online).

Results

Clonal Diversity

Redundant MLGs were always located within the same patch and considered as belonging to the same clone. Therefore, the total number of distinct genets considered was 23 and ranged from one in some of the smallest patches to six in the largest (Table 2), with 8 being sampled only once (35%) and 15 more than once (65%). The genotypic richness (*PD*) was 0.23, indicating that only 23% of the 100 ramets sampled belonged to distinct genotypes. However, the *p_{sex}* statistic showed that two out of the 100 ramets sampled (corresponding to MLGs K and O in Figure 1) might not be clonemates (*p_{sex}* > 0.05). Accordingly, the estimated genotypic richness (*PD*) was 0.25. The high dominance of genotypes within patches leads us to maintain the more conservative value of 0.23 for genotypic richness. Considering the whole population, we estimate the number of unique genets in the population to be around 46 to 50, with each genet comprising an average area of 290 m².

Genetic Diversity and Structure

A total of 23 alleles were amplified. The most frequent allele appeared at a frequency of 0.96, whereas 48% appeared at a frequency of between 0.3 and 0.62, and another 48% with a frequency below 0.2 (Table S1; see Supplementary Material online). The number of alleles per locus (*n_a*) was low, ranging from an average number of 1.43 to 2.29. The number

Table 2 Sample size (N), number of genets (N_G), percentage of polymorphic loci (PL), alleles per locus (n_a), mean allelic richness per locus (R_S), mean private allelic richness per locus (R_P), average expected (H_e) and observed (H_o) heterozygosities, inbreeding coefficients (F_{IS}). Standard deviations in parentheses. Diversity estimates are calculated at the ramet level

Patch	N	N_G	PL	n_a	R_S	R_P	H_o	H_e	F_{IS}
Patch 1	10	6	71.4	2.000 (0.816)	2.000 (0.816)	0.000 (0.000)	0.444 (0.346)	0.360 (0.244)	-0.251
Patch 2	10	3	71.4	1.714 (0.488)	1.700 (0.480)	0.000 (0.000)	0.571 (0.468)	0.314 (0.245)	-0.905
Patch 3	10	3	85.7	2.000 (0.816)	1.998 (0.814)	0.000 (0.000)	0.571 (0.406)	0.427 (0.211)	-0.364
Patch 4	10	2	85.7	2.000 (0.000)	1.998 (0.003)	0.143 (0.378)	0.571 (0.392)	0.349 (0.202)	-0.698
Patch 5	10	3	85.7	2.286 (0.756)	2.227 (0.718)	0.000 (0.000)	0.414 (0.426)	0.311 (0.230)	-0.356
Patch 6	10	2	71.4	2.143 (0.900)	2.143 (0.900)	0.000 (0.000)	0.357 (0.226)	0.395 (0.272)	0.100
Patch 7	10	1	42.9	1.429 (0.535)	1.429 (0.535)	0.000 (0.000)	0.429 (0.495)	0.226 (0.260)	-1.000
Patch 8	10	1	42.9	1.429 (0.535)	1.429 (0.535)	0.286 (0.488)	0.429 (0.495)	0.226 (0.260)	-1.000
Patch 9	10	1	42.9	1.429 (0.535)	1.429 (0.535)	0.143 (0.378)	0.429 (0.495)	0.226 (0.260)	-1.000
Patch 10	10	1	71.4	1.714 (0.488)	1.714 (0.375)	0.143 (0.378)	0.714 (0.452)	0.376 (0.238)	-1.000
Mean		2.3	67.1	1.814	1.807	0.072	0.493	0.321	-0.647
SD		1.6	17.9	0.316	0.307	0.101	0.109	0.074	0.401

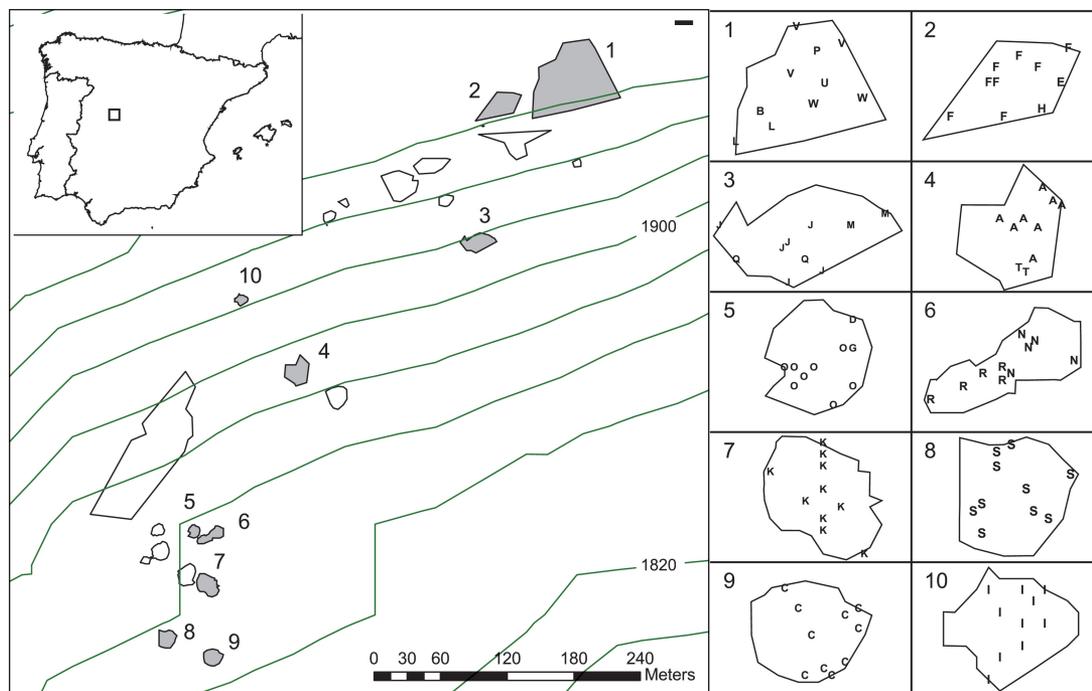


Figure 1. Patches of *P. rivis-martinezii* at La Serrota population. Disposition of genotypes within sampled patches (in gray) are represented on the right. Patches 5 to 9 are located in pastureland, whereas the rest are located in the shrubland.

of alleles and the mean allelic richness per locus (R_S) were highest on patch 5 and lowest on patches 7, 8, and 9. Private allelic richness (R_P) was almost insignificant for most patches. However, the number of polymorphic loci and the average heterozygosities were quite high, except in patches 7, 8, and 9, indicating a high distribution of genetic variation among patches (Table 2). The expected heterozygosity (H_e) ranged from 0.226 (patches 7, 8, 9) to 0.43 (patch 3). The observed heterozygosity (H_o), however, was significantly higher from H_e in almost all patches. Average expected heterozygosity for the population was 0.42 and average observed heterozygosity was 0.51. All patches showed surplus of heterozygosity,

except for patch 6, and the absence of most homozygote combinations for four of the seven loci.

In the FCA (Figure 2), the first factor (axis 1) essentially separates shrubland from pastureland (from left to right), with the exception of patch 2, which is closely related to pastureland patches, despite their geographic isolation (Figure 1). Consequently, when the upper level in the AMOVA was shrubland versus pastureland, *P. rivis-martinezii* samples were significantly differentiated within groups ($F_{ST} = 0.171$, $P = 0.0068$; Table 3), suggesting that habitat differences are a source of genetic differentiation. Indeed, shrubland patches showed significantly higher values for

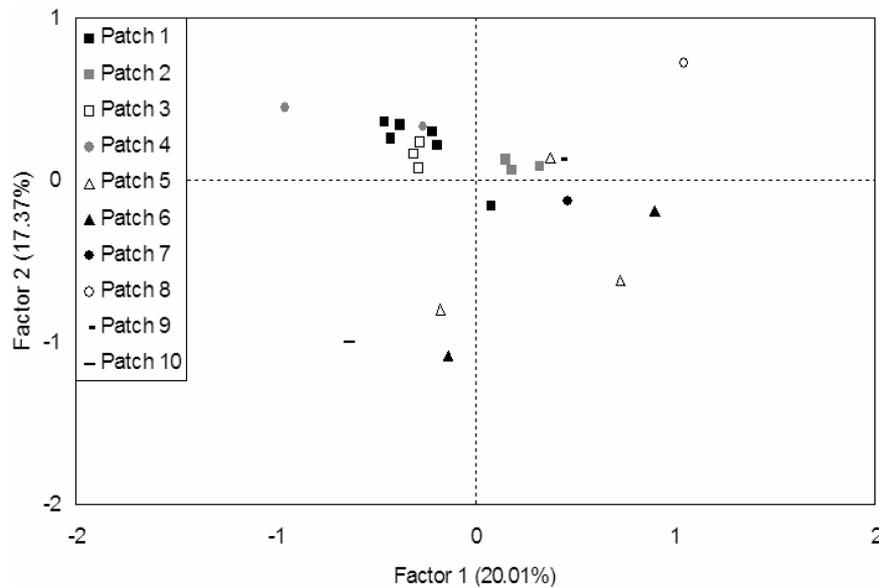


Figure 2. Factorial correspondence analysis representing genetic relationships between *P. rivas-martinezii* genets. The two first factors are plotted, explaining 20.01% (horizontal axis) and 17.37% (vertical axis) of the total variation, respectively. Patches 5 to 9 are located in pastureland, whereas the rest are located in the shrubland.

Table 3 Summary of ramet-level AMOVA for *P. rivas-martinezii*

Source of variation	d.f.	Sum of squares	Variance	% Variation	Fixation indices	P value
Among patches	9	143.83	0.75	40.09	0.401	<0.0001
Within patches	188	210.89	1.12	59.91		
Among habitats	1	46.45	0.35	17.09	0.171	0.0068
Among patches within habitats	8	97.37	0.56	27.55	0.332	<0.0001
Within patches	188	210.89	1.12	55.36	0.44637	<0.0001

observed heterozygosity ($M-W$, $Z = 2.61$, $P = 0.007$). The rest of the variation was mainly found within patches ($F = 0.446$, $P < 0.0001$; Table 3). Pairwise F_{ST} between patches showed significant differentiation ($P < 0.05$), with values ranging from 0.061 (between patches 5 and 7) to 0.617 (between patches 4 and 8) (Table S2; see Supplementary Material online).

Spatial Structure

The aggregation index indicated a clumped distribution for the population, with 73% of ramets sharing the same MLG with their closest neighbor ($Ac = 0.7305$, $P < 0.0001$, 1000 permutations) and a clonal sub-range of 35.85 m. On the whole, the spatial distribution of genets was mainly patchy, although some patches showed some level of intermingling. Thus, this perennial species combines both guerrilla and phalanx growth forms, with guerrilla predominating in the shrubland ($Ac = 0.5897$, $P < 0.0001$, 1000 permutations) and phalanx in the pastureland ($Ac = 0.8354$, $P < 0.0001$, 1000 permutations) (Figure 1). Furthermore, patches showed dominance of one large clone, because the proportional area

that genotypes would be occupying, according to the clonal repetitions observed, showed a right skewed distribution (Figure S4a; see Supplementary Material online), and the largest clone area was strongly correlated ($R^2 = 0.95$) to the total patch area within each patch (Figure S4b; see Supplementary Material online).

No relationship was observed between genetic distance (measured by F_{ST}) and geographic distance as indicated by the Mantel test ($\chi = -22.71$, $r = 0.211$, $P = 0.104$). The finer spatial autocorrelation analysis of microsatellite genotypes showed the clonal structure of the population. At the ramet level, a significant positive autocorrelation was found among ramets located up to 115 m. Beyond this distance coancestry values were not significantly different to 0 up to 140 m, after which a negative autocorrelation between ramets was observed (Figure 2a). At the genet level, results were very different and no significant correlation was observed along the fluctuating pattern observed (Figure 2b). Accordingly, the Sb value reflected the observed spatial autocorrelation at the ramet level ($Sb = 0.1643$), whereas its calculation at the genet level was not convenient, given the random distribution of genets in the sample.

Table 4 Results for the best Generalized Linear Mixed Models selected by the AIC procedure

Model 1		Response variable: number of flowers; random factor: patch		
Variables	Coeff. \pm SE	z value	P value	
Intercept	2.4993 \pm 0.2143	11.66	<0.001	
Branch	0.0842 \pm 0.0021	40.12	<0.001	
Shrub	-0.0229 \pm 0.0011	-20.05	<0.001	
Grazing	-1.0074 \pm 0.0639	-15.76	<0.001	
Model 2		Response variable: mean number of viable seeds per fruit; random factor: patch		
Variables	Coeff. \pm SE	z value	P value	
Intercept	-0.8924 \pm 0.3725	-2.40	0.017	
Distgenot	-0.0357 \pm 0.0174	-2.06	0.040	

Influence of Genetic and Environmental Factors on Fitness

Flower display ranged from 0 to 334 per ramet, with average flower production being 32.52 ± 5.73 per ramet. The estimated fruit set was $13.91\% \pm 1.66$. Out of the 554 seeds tested, only 11.19% were viable seeds. Seed set was $13.75\% \pm 2.33$, and viable seed set was $2.18\% \pm 0.63$. Shrub cover around ramets was on average $16.35\% \pm 2.51$. The overall proportion of ramets grazed by cattle was 21%, although grazing intensity was significantly higher (M-W, $\chi = -4.641$, $P < 0.001$) in grassland (40%) than in shrubland (2%) patches. Similarly, ramet density was significantly higher (ANOVA, $F = 35.545$, $P < 0.001$) in grassland (38.12 ± 2.69) than in shrubland (15.36 ± 2.69) patches.

No significant correlation was found between the number of genotypes per patch and unfavorable environmental conditions (shrub canopy closure and grazing) (Table S4a; see Supplementary Material online). Although the differential intensity of adverse environmental conditions among patches may not be strong enough to show a decrease in the number of genotypes, this effect cannot be discarded at the population level. However, flower production was positively correlated with clone size, whereas fruit set and seed viability were negatively correlated with clone size (Table S4b; see Supplementary Material online).

The reproductive components (number of flowers and mean viable seeds) were correlated with genetic and environmental variables (Table 4). First, once the difference in the number of branches was taken into account, an increase in shrub cover and the presence of grazing both decreased flower display. Second, the main predictor for the mean number of viable seeds per fruit was the increasing distance to a different genotype, which lowered seed viability.

Discussion

High Genetic Diversity Despite Clonality

The existence of very few and unique genotypes within patches is a common result for clonal plant species (Loveless and Hamrick 1984; Ellstrand and Roose 1987, Arnaud-Haond et al. 2007; Honnay and Jacquemyn 2008). The level

of genotypic richness found in *P. rivas-martinezii* ($PD = 0.23$) was low compared to that shown by other clonal species. Indeed, reviews of SSRs on clonal species by Honnay and Jacquemyn (2008) yielded a mean value of $PD = 0.44 \pm 0.08$ for all species included.

Clonal species have been demonstrated to be as genetically diverse as nonclonal species (Ellstrand and Roose 1987; Widén et al. 1994; Hamrick and Godt 1996; Richards et al. 2004). *Pseudomisopates rivas-martinezii* is expected to be more genetically diverse when compared to species with similar life histories (Hamrick and Godt 1996), despite these results were obtained using allozymes. Closely related self-incompatible species of *Antirrhinum* in the Iberian Peninsula are less genetically diverse, with about 50% of polymorphic allozyme loci and a expected heterozygosity of 0.029 to 0.470 (Jiménez et al. 2002; Carrió et al. 2010). Again, the heterozygote maintainance and the absence of most homozygote combinations is a common characteristic of clonal and self-incompatible species (Evans et al. 2003; Halkett et al. 2005; Stoeckel et al. 2006). In addition, this species fulfils the assumption of monophyly, and therefore loci polymorphism due to hybridization is ruled out (Carrió et al. 2010). Certainly, diverse colonizing cohorts and high genet persistence (Eriksson 1993; Watkinson and Powell 1993) must be maintaining the population even if seed recruitment is low (Watkinson and Powell 1993).

The uniqueness of genotypes among patches is reflected on the strong genetic differentiation, as indicated by the fixation index (Table 3) and the pairwise F_{ST} (Table S2; see Supplementary Material online), although differentiation would be more due to differences in allele frequencies rather than unique alleles (Table 2). Differentiation among patches could be due to past fragmentation of the population and colonization from seed, whereas the stronger differentiation within patches points toward a limited dispersal of both pollen and seeds. In addition, differentiation between pastureland and shrubland could be the result of habitat differences, intense grazing, and growth form. Pastureland patches have phalanx ramets that are more affected by grazing. Whereas, the spreading guerrilla ramets in the shrubland enhance successful pollination (Handel 1985), as shown by the significantly higher heterozygosity in shrubland patches.

Strong Genetic Spatial Structure as a Result of Clonality

Clonal reproduction resulted in a clumped distribution of ramets overall. Hence the population showed dominance of a few large clones and a majority of either small or intermediate clones (Figure S4; see Supplementary Material online). The lack of isolation by distance suggests limited gene flow between patches. The finer scale spatial structure analysis, however, revealed strong nonrandom mating among ramets up to a distance of 115 m (Figure 3a) but complete random mating among genets (Figure 3b). The strong SGS observed is the result of intense clonal growth and an indication of a long time since the last disturbance took place. The random genetic structure among genets throughout the area is not too common, although it has been observed in many oak species (Alfonso-Corrado et al. 2004), probably due to their self-incompatible and wind-pollinated systems. This structure may be related to the self-incompatible mating system of the species, by which flowers need necessarily to be fecundated by pollen from a different genet. Thus, seed viability is likely to be affected by this SGS of clones, which would lower compatible mate availability by proximity. These results are congruent with the pollen quality limitation reported by previous studies (Amat et al. 2011).

Advantages and Disadvantages of Clonality

Our results support the hypothesis that fitness could be eventually reduced by environmental conditions. Indeed, shrub canopy closure and herbivory seem to reduce the investment of ramets into sexual reproduction and could be enhancing clonality, which would in turn reduce mate availability due to the SGS created by clones. Under these circumstances, plants would reduce their investment into sexual reproduction and enhance clonal spread. Translocation of resources and reproductive investment is commonplace between ramets in many clonal species (D'Hertefeldt and Jónsdóttir 1994). In some cases, flowering individuals may

only be observed at initial stages or originate from plants that have persisted in shaded places until gap formation (Kanno and Seiwa 2004). Many clonal herbaceous forest plants show increased sexual vigor in gaps. For instance, management practices that affect the degree of canopy closure affected plant density and sexual reproduction in *Maianthemum dilatatum* (Lezberg et al. 2001); differences in fruit size and seed number in *Trillium erectum* were associated with a lower carbon allocation in populations with more intense canopy closure (Routhier and Lapointe 2002); and suppressed flowering and monoclonal populations were reported in closed canopy habitats in *Uvularia perfoliata* (Kudoh et al. 1999). In addition, in many clonal species, grazing and mowing prevent sexual reproduction and resources are allocated toward clonal spread (Kerley et al. 1993; Schaal and Leverich 1996). The higher ramet density on intensely grazed patches of *P. rivis-martinezii* could be a response to grazing. Moreover, herbivores, through selectively feeding on herbaceous species, are considered to increase the resources available to woody species, increasing shrub cover under grazing (Walker et al. 1981; Skarpe 1990). Under these circumstances, clones may gain a relative benefit in terms of clonal spread, because they are more likely to persist by clonal reproduction under less favorable environmental conditions.

At the same time, the increased survival opportunity created by clonality would diminish mate availability due to the SGS created by clones. Evidence shows that this must be a common situation in this species, because although fruit set was higher than in other clonal species (Eriksson and Bremer 1993; Honnay et al. 2006), the percentage of viable seeds was very low. Accordingly, clone size significantly increased flower display, but decreased both fruit set and seed viability. Larger clones would produce more flowers, because they may have more resources available that can be invested into a higher number of meristems (e.g., *Lolium perenne* in Thiele et al. 2009), whereas the SGS created by large clones seems to decrease reproductive success due to lowered mate availability. At a finer scale, results indicate that the mean number of viable

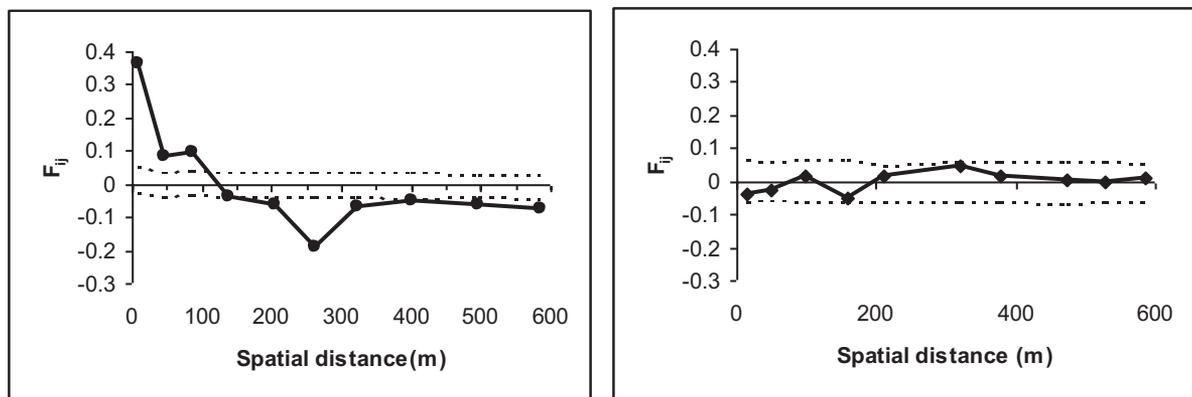


Figure 3. (a) Ramet level correlogram showing kinship coefficients of *P. rivis-martinezii* at different spatial distance classes. (b) Genet-level correlogram produced with central coordinates for redundant genets. Dotted lines delimit 95% confidence intervals around the null hypothesis of randomly spatially distributed genets.

seeds decreases by larger distances to ramets with a different genotype, indicating that the SGS created by clonal growth imposes a physical mating barrier as observed in other clonal species, for example, *Rubus saxatilis* (Eriksson and Bremer 1993), *Calystegia collina* (Wolf et al. 2000), *Maianthemum bifolium* (Honnay et al. 2006), and *Convallaria keiskei* (Araki et al. 2007). Thus, these results support previous hand pollination studies and floral visitor observations that showed that the low sexual reproductive success in *P. rivas-martinezii* was mainly a result of pollen quality limitation, probably due to low mate availability, and secondarily of pollen quantity limitation, due to inefficient pollination (Amat et al. 2011).

Although seed production is often emphasized as being a major fitness component, clonal growth should also be considered a measure of fitness in clonal plants, because it affects fitness through genet persistence and seed production, as well as through the genotypic selection of better-adapted ramets (Pan and Price 2002). Population dynamics of clonal species is deeply related to genet size and age structure (de Witte and Stöcklin 2010), together with the reproductive compensation across seasons (Goodwillie 1999; Larson and Barrett 2000). Deeper knowledge of population dynamics of *P. rivas-martinezii*, considering both the effect of genet persistence and sexual reproduction compensation over time, would better clarify the effects of clonality in this species, although the high genetic diversity detected gives insights into the health of this population and the suitability of a mixed reproductive strategy for this species.

Habitat Disturbance and Conservation

The ultimate goal of conservation is to ensure the survival of populations, while maintaining their evolutionary potential (Frankham et al. 2002). For rare and endangered species, this has relied on conserving their ecosystems (Franklin 1993; Lindenmayer et al. 2007). However, in disturbance-prone species, conservation strategies might require soil disturbance and opening of the canopy, as assessed by traditional management practices, for successful seedling recruitment.

Genetic diversity allows populations to adapt to a changing environment and is vital for assessing suitable conservation strategies (Hamrick 1983; Falk and Holsinger 1991; Frankham et al. 2002). Increasing the number of individuals per population would be effective in preventing genetic loss and the chance of local extinction by stochasticity. However, in the case of clonal species that germinate in response to disturbance and are later overgrown by shrubs, resprouting and seeding play a crucial role (Keeley and Fotheringham 2000; Ojeda Copete et al. 2005). Besides, high heterozygosity observed in this population suggests that it is unlikely to be suffering from inbreeding depression and that it might be valuable for conservation, although erosion of allelic richness at self-incompatibility loci may be limiting the reproductive capacity of populations (Young et al. 1999).

Clonality is limited by disturbance (Silvertown 2008), so that many plants in disturbance-prone ecosystems show a mixed reproductive system that allows them to adapt to environmental stochasticity. *Pseudomisopates rivas-martinezii*

inhabits the clearly anthropogenic landscape of Gredos Range, which includes a combination of forest patches, pastures, and widespread shrublands, mostly generated during the transition between the 17th and 18th centuries AD, when forest management activities, including fire and intense grazing, caused the progressive deforestation and consequently the expansion of the current fire-prone shrubland (López-Merino et al. 2009). Accordingly, germination studies have shown that the species exhibits specificity to fire. A positive response to the effects of fire, both directly (79% higher germinability after ash treatments) and indirectly (higher resprouting in burned sites and 74% lower germinability in darkness), has been observed (Amat et al., unpublished data). Moreover, seed viability varies across years, so that certain compensation could be taking place (Amat et al., unpublished data). Therefore, despite being critically endangered, clonality probably represents the best strategy for the survival of this species till the next disturbance takes place.

Fire is a natural process in many Mediterranean ecosystems and has been implemented successfully in managing endangered species (Fensham and Fairfax 1996), as in the iconic *Sequoiadendron giganteum* (Swetnam et al. 2009). Nevertheless, incorporating fire as a management tool for increasing the abundance of an endangered or rare species must be taken with caution because fire may negatively affect plant communities as a whole and the precise effects on the species must be monitored (Pendergrass et al. 1999). Consequently, further knowledge on population dynamics and response to the disturbance caused by fire in *P. rivas-martinezii* is essential for conservation.

In contrast, disturbance by grazing affects complete ramets and patches. Recruitment will remain suppressed and populations threatened by lack of replacement and loss of genetic flexibility. To maintain *P. rivas-martinezii* populations in the long term, lengthy periods of grazing rest and controlled grazing pressure will be necessary to allow both new recruits and mature individuals to reproduce.

Concluding Remarks

In conclusion, the species exhibits a mixed reproduction system, which allows it to benefit from different strategies and to escape from environmental stochasticity. Sexual reproduction is the main factor determining the formation of new genets and is important at determining the population genetic structure. In turn, clonal growth is one of the main factors determining population growth and SGS. Although seed viability is affected by the SGS of clones, which lowers compatible mate availability, seed production may be enough to compensate across years as shown by the maintenance of genetic diversity. Furthermore, under adverse environmental conditions of canopy closure and intense grazing in which sexual reproduction and genetic diversity may be reduced, clonality allows *P. rivas-martinezii* to persist and escape extinction in a successional shrubland environment.

Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

Funding

This research was supported by the Spanish General Direction of Scientific and Technical Research (DGICYT) through the projects CGL2009-1003 and FGCSIC-2010. M.E.A. was supported by an FPU fellowship (AP2005-0160) from the Spanish Ministry of Education, which allowed her visit to The Open University, where part of this project was carried out.

Acknowledgments

The authors thank Bernardo García Muñoz for information on populations; Gonzalo García-Baquero and Jorge Martínez for field assistance; Alan Forrest, Andrew Hudson, Yvette Wilson, and Brendan Davies for SSR primers; Pamela Biss and Andrea Costa for lab assistance, and Liliána Davalos, Xavier Picó, and Miriam Heuertz for analytical advice. The Consejería de Medio Ambiente of the Junta de Castilla y León granted permission to work in Gredos Regional Park.

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Received October 31, 2012; First decision December 12, 2012;
Accepted June 9, 2013

Corresponding Editor: F. Andrew Jones

SUPPLEMENTARY MATERIAL

Table S1. Number of alleles, heterozygosity and F_{is} at each microsatellite locus for *P. rivasmartinezii* patches in the study site.

Locus	Number of alleles	H_{obs}	H_{sn}	F_{is}
MSAT 35	2	0.072727	0.079257	0.082386
MSAT 53	2	0.500000	0.509174	0.018018
MSAT 61	5	0.759259	0.699846	-0.08489
MSAT 63A	4	0.690909	0.639257	-0.0808
MSAT 63B	5	0.709091	0.64686	-0.0962
MSAT 69	3	0.333333	0.599881	0.444334
MSAT 77	2	0.333333	0.475738	0.299334

Table S2. Patch pairwise differentiation. All F_{st} values are significant (p-value <0.0001).

	PATCH 1	PATCH 2	PATCH 3	PATCH 4	PATCH 5	PATCH 6	PATCH 7	PATCH 8	PATCH 9	PATCH 10
PATCH 1	0									
PATCH 2	0.277	0								
PATCH 3	0.148	0.255	0							
PATCH 4	0.204	0.407	0.146	0						
PATCH 5	0.361	0.271	0.293	0.476	0					
PATCH 6	0.302	0.378	0.323	0.474	0.297	0				
PATCH 7	0.448	0.365	0.374	0.563	0.061	0.380	0			
PATCH 8	0.465	0.460	0.443	0.617	0.384	0.380	0.426	0		
PATCH 9	0.536	0.307	0.374	0.548	0.277	0.474	0.368	0.549	0	
PATCH 10	0.312	0.396	0.315	0.374	0.483	0.383	0.532	0.532	0.557	0

Table S3. Results for the Generalized Linear Mixed Models relating *Pseudomisopates rivasmartinezii* reproductive variables (a: flower display; b: mean number of viable seeds) to environmental and genetic factors: (1) SH: shrub, (2) GR: grazing, (3) FS: fruitset, (4) FT: number of fruits, (5) FL: number of flowers, (6) DM: diameter, (7) RT: ramet density, (8) DG: minimum distance to a different MLG, (9) DP: minimum distance to a different patch. Significant variables ($p < 0.05$) in bold.

(a) FLOWER DISPLAY: Model selection on phenotypic and environmental variables.

MODEL	VARIABLES	K	LOGLIK	AIC
1	BR+SH+GZ	3	-843.1	1696
2	BR+SH	2	-986.7	1981
3	DM+SH+GZ	3	-1066	2143
4	BR+GZ	2	-1120	2248
5	DM+SH	2	-1173	2353
6	BR+DG	2	-1244	2496
7	BR	1	-1246	2498
8	DM+GZ	2	-1369	2746
9	DM+DG	2	-1461	2930
10	DM	1	-1470	2945
11	SH	1	-1707	3419
12	GR	1	-1793	3591
13	DG	1	-1849	3703
14	RT	1	-1861	3728

(b) MEAN VIABLE SEEDS: Model selection on genetic, reproductive and environmental variables.

MODEL	VARIABLES	K	LOGLIK	AIC
1	DG	1	-52.9321	107.8642
2	DG+SH	2	-52.1962	108.3924
3	DG+BR	2	-52.381	108.762
4	DG+FR	2	-52.762	109.524
5	DG+FL	2	-52.8254	109.6508
6	DG+FL+SH	3	-51.9122	109.8244
7	DG+FL+BR	3	-52.3669	110.7338
8	DP	1	-54.4165	110.833
9	SH	1	-54.8879	111.7758
10	GR	1	-54.9408	111.8816
11	FS	1	-55.0564	112.1128
12	FT	1	-55.1965	112.393
13	BR	1	-55.2886	112.5772
14	FL	1	-55.2948	112.5896
15	RT	1	-55.4109	112.8218
16	DM	1	-55.5771	113.1542

Table S4. Generalized Linear Models exploring the effects of the number of genotypes per patch and the size of clones.

(a) Models relating number of genotypes per patch to environmental variables (grazing and shrub cover).

MODEL 1	Response variable: Number of genotypes		
	Distribution: Poisson		
Variables	Coeff ± Std Error	z value	p-value
Intercept	1.1335 ± 0.2578	4.398	<0.001
Grazing	-1.7688 ± 1.1243	-1.573	0.116

MODEL 2	Response variable: Number of genotypes		
	Distribution: Poisson		
Variables	Coeff ± Std Error	z value	p-value
Intercept	0.33584 ± 0.44324	0.758	0.449
Shrub cover	0.02784 ± 0.02021	1.378	0.168

(b) Models relating reproductive variables (flower production, fruit set and seed viability) to estimated genotype area.

MODEL 1	Response variable: Number of flowers		
	Family: Poisson		
Variables	Coeff ± Std Error	z value	p-value
Intercept	3.298e+00 ± 4.845e-02	68.08	<0.001
Genotype area	1.462e-03 ± 9.365e-05	15.62	<0.001

MODEL 2	Response variable: Fruit set		
	Family: Poisson		
Variables	Coeff ± Std Error	z value	p-value
Intercept	2.6342 ± 0.0864	30.487	<0.001
Genotype area	-0.0009 ± 0.0003	-3.192	0.001

MODEL 3	Response variable: Seed viability (%)		
	Family: Poisson		
Variables	Coeff ± Std Error	z value	p-value
Intercept	2.5468 ± 0.1203	21.169	<0.001
Genotype area	-0.0044 ± 0.0007	-6.112	<0.001

Figure S1. Box plot describing the genotypic resolution of microsatellites in *Pseudomisopates rivas-martinezii* containing 100 sampling units genotyped using 7 microsatellites (1000 permutations). Box edges show the minimum and maximum number of genotypes, and the central line shows the average number of genotypes identified. The graph shows that a set of seven loci allows an accurate determination of the number of genotypes in the sample.

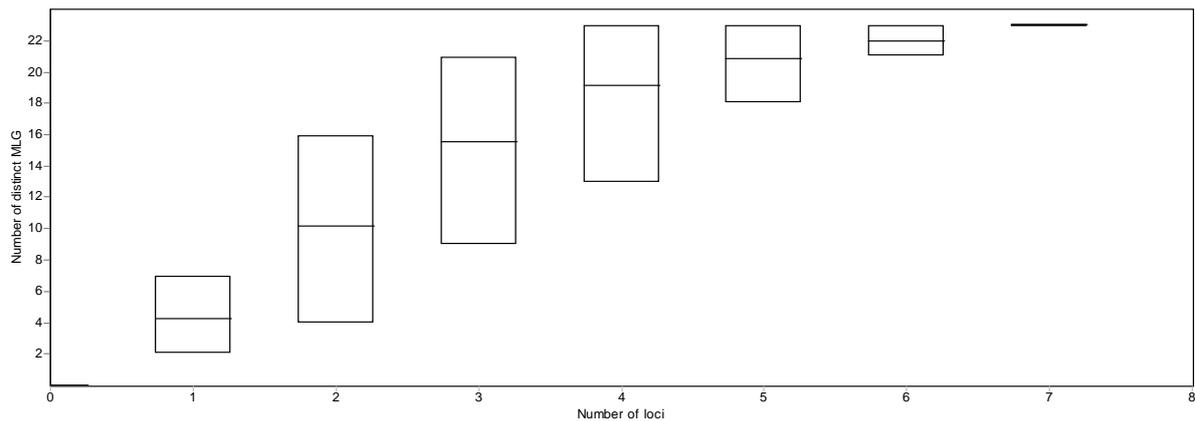


Figure S2. Frequency distribution of the pairwise number of alleles differences between MLGs for *Pseudomisopates rivas-martinezii* (100 permutations). The dashed line in the adult distribution represents the p_{sex} threshold below which identical MLGs would have originated from the same zygote.

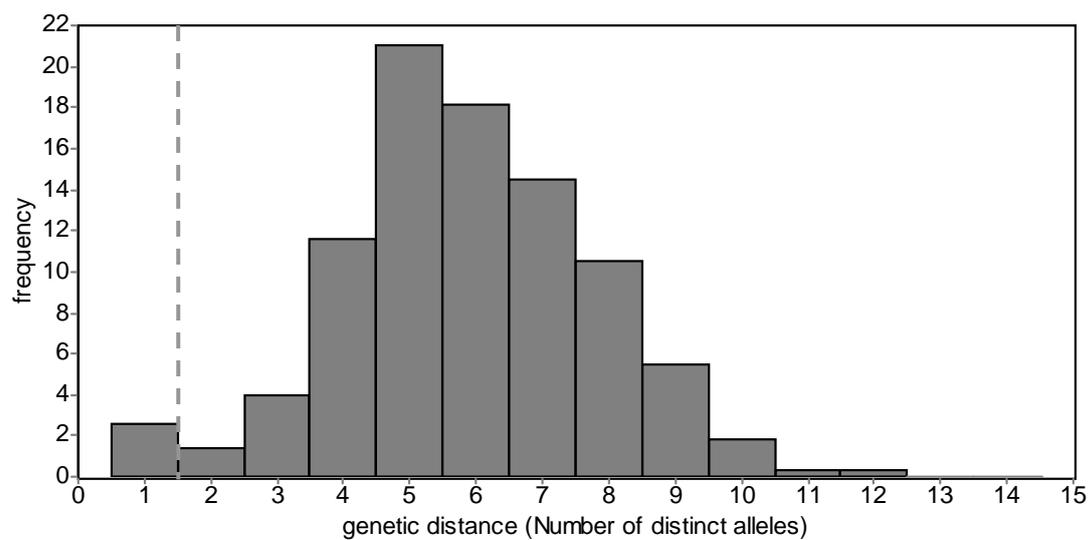
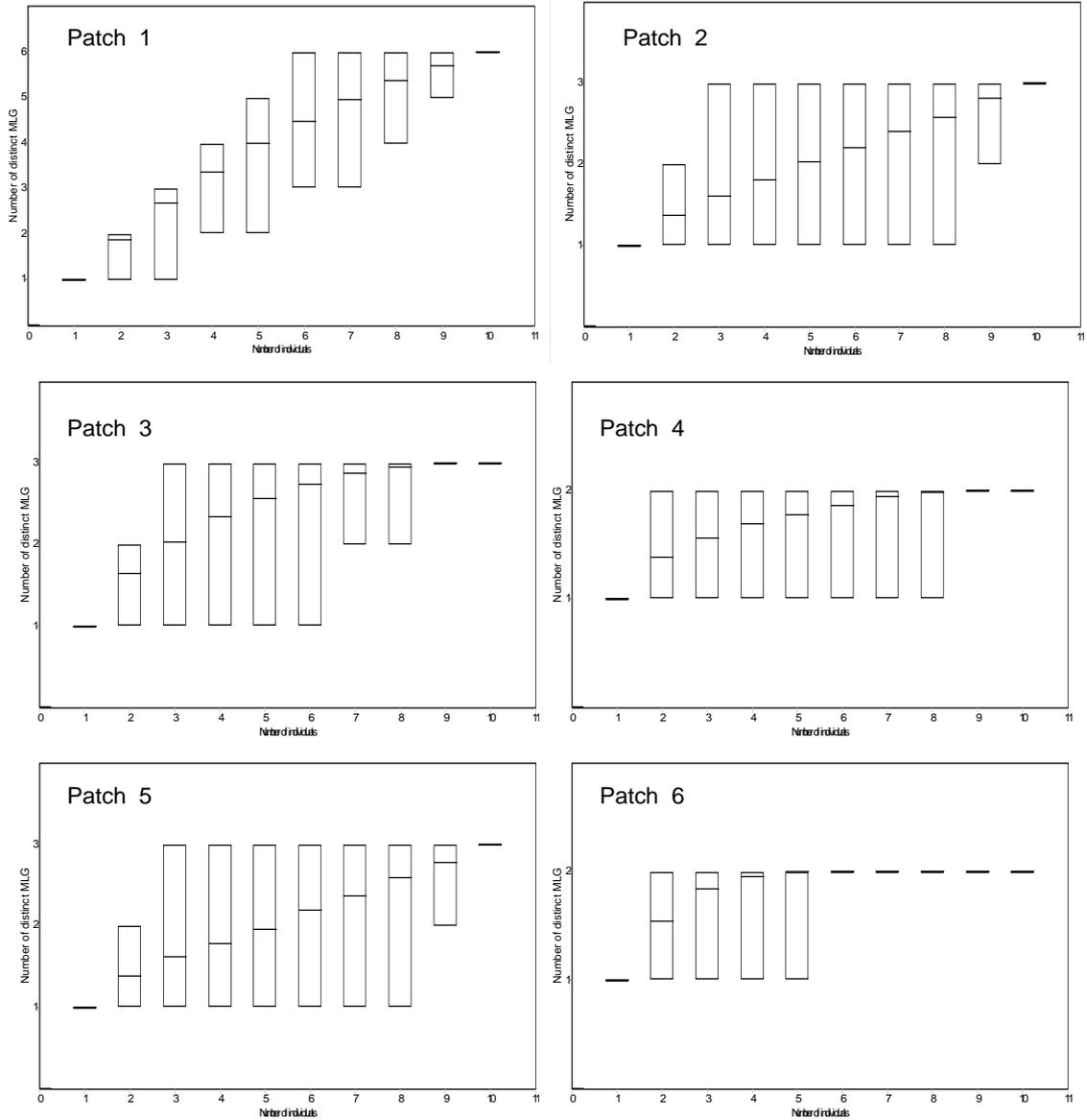


Figure S3. Box plots representing the number of MLGs of *Pseudomisopates rivas-martinezii* detected with an increasing sampling effort of up to 10 samples per patch (100 permutations). Box edges show the minimum and maximum number of genotypes, and the central line shows the average number of genotypes identified. The graphs show that a set of ten samples per patch was sufficient to detect the number of genotypes present.



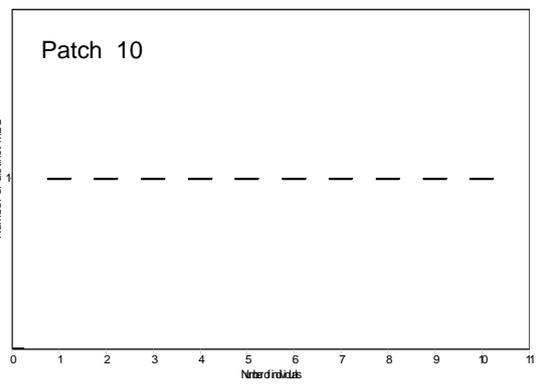
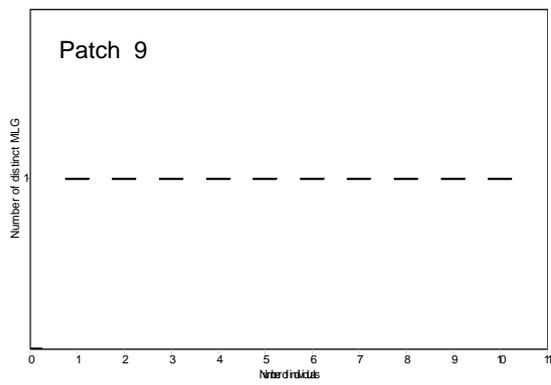
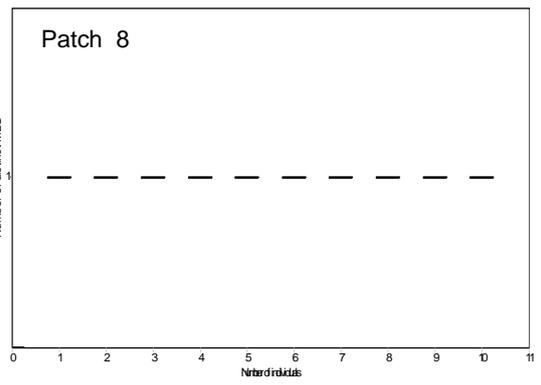
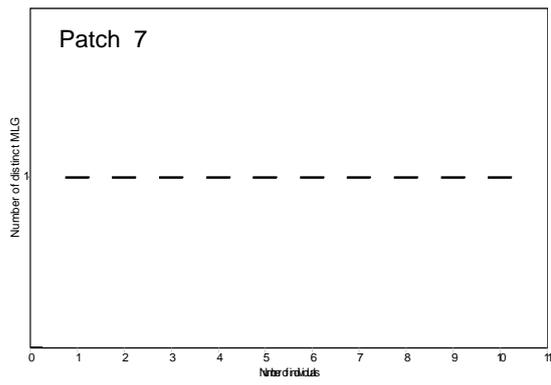


Figure S4. (a) Proportional area potentially occupied by the different genotypes, according to the clonal repetitions observed in the sample. A-W: genotype names. (b) Scatterplot showing the relation between patch area and the area of the largest genet per patch.

