Resistance of an edaphic-island specialist to anthropogenic-driven fragmentation

Alfredo García-Fernández1*, José M. Iriondo1, Bernardo de Haro Reyes2 and Adrián Escudero1

1Dept. Biología y Geología, Física y Química Inorgánica, ESCET, Universidad Rey Juan Carlos, C/ Tulipan SN, 28933 Mostoles, Spain
2School of Applied Sciences, University of Huddersfield, Huddersfield HD1 3DH, UK

Received: 14 June 2017  Editorial decision: 26 November 2017  Accepted: 8 December 2017  Published: 9 December 2017

Associate Editor: Philippine Vergeer

Citation: García-Fernández A, Iriondo JM, de Haro Reyes B, Escudero A. 2018. Resistance of an edaphic-island specialist to anthropogenic-driven fragmentation. AoB PLANTS 10: plx072; doi: 10.1093/aobpla/plx072

Abstract. Fragmentation is one of the most important human-induced threats to biodiversity. Linear infrastructures, together with agriculture intensification, alter migration patterns, inducing isolation and/or affecting the connectivity between populations. The combined effect of these drivers has, as far as we know, never been explored. A population genetics approach was proposed to assess the effects of both drivers in an edaphic specialist. We selected a fragmented scenario of gypsum soil habitats, within an agricultural matrix divided by a highway to assess the impact of fragmentation on the genetic structure of the edaphic specialist, Helianthemum squamatum. For each patch, connectivity values, soil composition and plant densities were estimated. Microsatellite markers were used to evaluate the genetic diversity of each fragment, gene flow between patches and global genetic structure. Sampled patches showed similar values for the genetic parameters, suggesting the absence of a defined genetic structure and scarce influence of these two fragmentation drivers on gene flow. No association between ecological characteristics of the fragments and genetic features was found, although population density was correlated with inbreeding coefficient. Species biology and population dynamics are essential factors to understand the effects of fragmentation. Since the studied species is an edaphic specialist, its evolutionary history has taken place in an island-like scenario and, thus, human-driven fragmentation may have only marginal effects on its genetic structure. The results also outline the importance of maintaining a moderate population size within each fragment to avoid the potential genetic pernicious effects.

Keywords: Agricultural matrix; fragmentation; gypsum soils; Helianthemum squamatum; highway; landscape genetics.

Introduction

Habitat fragmentation is one of the major threats to global biodiversity (Fahrig 2003). Its effects are widespread, poorly predictable and involve all kinds of organisms (Debinski and Holt 2000). Fragmentation modifies gene flow among populations and reduces the habitat available to the species, disturbing the connectivity among remnants, increasing edge effects and decreasing habitat quality (see Fahrig 2003). However, the ecological implications of fragmentation are far from being fully understood since it may generate contrasting biological responses making outcomes difficult to predict (Ewers and Didham 2006). Thus, though some species

*Corresponding author’s e-mail address: alfredo.garcia@urjc.es

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may be drastically harmed in fragmented landscapes, some may be totally unaffected and, still some others may even respond positively (Fahrig 2002), suggesting that the consequences of fragmentation are idiosyncratic and dependent on specific processes (Rabasa et al. 2005; Matesanz et al. 2015).

Unfortunately, the pressure on gypsum ecosystems is increasing in certain areas (Escudero 2009; Matesanz et al. 2009) and it is very frequently the simultaneous incidence of several factors that drive fragmentation (Pueyo et al. 2008; Matesanz et al. 2009). For instance, relatively stabilized fragmented landscapes originated by historical agricultural practices are more recently being rapidly transformed by the construction of highways or other linear infrastructures (Storfer et al. 2006).

The potential impacts of a transportation infrastructure over the landscapes and ecosystems have been widely reviewed (e.g. Debinski and Holt 2000), including negative and positive effects on biodiversity. Gene flow between the two sides of the infrastructure is likely to be disturbed due to barrier or corridor effects (Holderegger and Di Giulio 2010; Landguth et al. 2010). On the other hand, the changes associated to the creation of novel ecosystems at roadsides may boost gene flow along the sides of the highway by providing new step stones (Suárez-Esteban et al. 2013). Both processes can sharply modify the previously existing connectivity pattern between remnant fragments in an agriculture landscape where shrinking islands can increase their isolation (Ewers and Didham 2006).

Landscape genetics combines principles of landscape ecology with population genetics hypotheses and the information provided by molecular markers (Holderegger and Wagner 2006). It offers a multidisciplinary approach to study fragmentation effects from a holistic perspective not centred on specific life stages and processes (dispersal, pollination, recruitment; Manel et al. 2003). By using these tools it is possible to evaluate the connectivity between patches and the existence of genetic barriers in the studied organisms (Manel et al. 2003). The increasing numbers of studies of landscape genetics, including a vast variety of organisms and molecular markers (see review Storfer et al. 2010), are improving our knowledge of the biological implications of human-driven fragmentation and recommend its use for conservation purposes (Fahrig 2003; Storfer et al. 2006).

Among the knowledge gaps that remain on the consequences of fragmentation in plant populations, the effect of fragmentation on edaphic gypsum specialists, whose evolution has mostly taken place in island-like scenarios, has hardly been studied (but see Matesanz et al. 2015). This is surprising since this type of special soil archipelago is a perfect natural laboratory in which to evaluate the consequences of fragmentation in inland territories (Escudero et al. 2015) and test hypotheses on gene flow between habitat remnants (Martínez-Nieto et al. 2013; Gómez-Fernández et al. 2016).

This knowledge is demanded in particular since gypsum ecosystems are considered among the most threatened habitats in Europe (EU, Habitat Directive), their flora including a large number of narrow endemics and rare species (Olano et al. 2005), and human-driven activities are exacerbating natural fragmentation by reducing fragment size and connectivity through agricultural intensification, quarrying exploitation or other mechanisms of perturbation (Fahrig 2001; Fahrig 2003; Tschamkute et al. 2005).

Our working hypothesis is that, having evolved in island-like evolutionary scenarios, widespread gypsum soil specialists such as *Helianthemum squamatum* (see Palacio et al. 2007) are able to thrive in man-made, fragmented remnants subject to great isolation. This is paradoxical since most gypsophytes have evolved specific traits to favour the recruitment of the progeny in the near vicinity of their mother plants limiting the gene flow among gypsum islands. It is probable that this ability to connect gypsum islands involved the existence of a very generalist pollination strategy (Aragón and Escudero 2008) and sporadic but efficient long-distance dispersal (LDD) events (e.g. Matesanz et al. 2015; Gómez-Fernández et al. 2016).

The aim of this study was to assess how an edaphic island specialist, which has evolved to persist in naturally fragmented landscapes (Matesanz et al. 2009; Escudero et al. 2015; Sánchez et al. 2017), coops with an increase in the intensity of fragmentation due to anthropic drivers. For this purpose, we studied populations of *H. squamatum*, a widespread and locally abundant Iberian gypsum specialist. The selected territory has a long history of fragmentation driven by agriculture and, more recently (i.e. 25–30 years ago), by the construction of a highway with dense traffic (Munoz 2003). We hypothesized that the genetic effects of anthropogenic fragmentation would be buffered by the life history traits of *H. squamatum* resulting from adaptation to naturally fragmented landscapes. Specifically, we asked (i) Are populations in different remnants genetically differentiated? (ii) If so, has gene flow limitation generated by human-induced fragmentation originated a pattern of isolation by distance (IBD) in the metapopulation? (iii) What impact does the presence of an active highway have on the genetic structure and genetic diversity of the populations?
Methods

Study plant and population selection

*Helianthemum squamatum* (Cistaceae) is a small perennial shrub (10–40 cm) that inhabits the driest gypsum outcrops of the Iberian Peninsula. It is a short-lived species (up to 10 years), diploid, with hermaphroditic and partially self-compatible flowers (Aragón and Escudero 2008). The flowering period lasts from May to August and flowers are pollinated by *Bombylius* spp. and other generalist pollinators (Aragón and Escudero 2008). Seeds do not show specific structures for dispersal but contain a mucilaginous coat that favours its adhesion to the soil and, thereby, promotes germination close to mother plants. *Helianthemum squamatum* is a diagnostic and frequently occurring species in Iberian gypsum communities (details of the plant community composition can be found in Olano et al. 2005).

We selected a homogeneous semi-arid gypsum landscape in south-east of Madrid (central Spain; Fig. 1), crossed by a highway with high-traffic volume (four traffic lanes, 50,000–80,000 vehicles per day, Spanish Ministry of Fomento 2013). The landscape is composed of gypsum vegetation fragments that are interspersed throughout a matrix of extensive non-irrigated crop-lands. The study area covers a rectangular area of 40 km² (~13 km long and 3 km wide). The highway crosses the study area from north-west to south-east, dividing the landscape into two comparable halves (Fig. 1). In the study area, all gypsum vegetation remnants were visited and 21 of them were haphazardly selected, covering gradients of population size and geographic fragment isolation. When available, we selected up to 20 individuals of *H. squamatum* in each patch, keeping a minimum distance of 10 m between individuals. From each individual, three to five fresh young leaves were collected. The final number of individuals sampled in each fragment is shown in Table 1.

Fragment characterization and connectivity estimations

We performed a physical-chemical soil analysis to characterize habitat quality and test for differences in soil composition that could affect plant fitness and density (Matesanz et al. 2009). In each fragment, 10 cylindrical cores of soil (6 cm in diameter by at least 10 cm deep) were obtained, five from exposed soil and a further five beneath vegetated clumps. For each soil sample, total nitrogen (N), phosphorus (P), potassium (K), organic carbon, pH, conductivity and glucosidase and phosphatase enzyme activities were measured. Soil samples were sieved (2 mm mesh) and air-dried under lab conditions for 1 month. We then estimated soil organic carbon by colorimetry after oxidation with K₂Cr₂O₇ and H₂SO₄ (Anderson and Ingram 1989) and total amount of N, P and K on a SKALAR++ San Analyzer (Skalar, Breda, The Netherlands) after digestion in H₂SO₄ and Kjedahl's catalyst.

For each selected fragment, we also measured different landscape variables related to connectivity and fragmentation. Thus, distance from both the centre and the nearest border of the fragment to the highway was...
measured. Furthermore, total fragment area and perimeter were measured using aerial photographs from the year 2011. The connectivity of each fragment was calculated according to the index described by Tremlová and Münzbergová (2007):

\[
C_i = \log_{10} \sum_{k=1}^{n} A_k / d_{ik}, \quad i \neq k
\]

where \(C_i\) is referred to the connectivity of the fragment \(i\), \(n\) is the total number of fragments around the target fragment that are included within a 500 m radius, \(A_k\) is the area of fragment \(k\) and \(d_{ik}\) is the minimum distance between fragments \(i\) and \(k\). A radius of 500 m was taken into consideration for this study using a conservative approach, because generalist pollinators are able to forage many sources of pollen within a unique fragment.

The population density of \(H.\ squamatum\) in each fragment was estimated by marking out two linear transects 1 m wide and up to 250 m long and counting the individuals of \(H.\ squamatum\) found.

### DNA extraction and PCR conditions

DNA extraction was carried out using a Speedtools plant DNA extraction kit (Biotools, Madrid, Spain) following the recommended protocol with 60 mg of dried tissue for each individual. Eight nuclear microsatellite loci, specifically designed for \(H.\ squamatum\) (García-Fernández et al. 2014), were amplified in a PCR mix containing 3 pmol each of the forward (labelled with fluorescence dye) and reverse primers (unlabelled), 1× Taq buffer, 0.2 mM of each dNTP, 2 mM MgCl₂, and 1 U Taq DNA polymerase (Biotools) and 20 ng of template DNA (final volume 20 μL). The PCR program consisted of one initial

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**Table 1.** Populations of *Helianthemum squamatum* sampled in the genetic study. Population number corresponds to the fragment ID of the study area. Letters in brackets denote its location on either side of the highway: SW, south-west; NE, north-east. For each population, number of individuals sampled, allelic richness, number of private alleles, expected \((H_e)\) and observed \((H_o)\) heterozygosity, number of fixed alleles, inbreeding coefficient \((F_{IS})\) estimated with IN2est, number of loci that deviated from Hardy–Weinberg (HW) equilibrium and the sum of migration rates to other patches, estimated with BayesAss, are shown.

| Population | Individuals sampled | Allelic richness (private alleles) | \(H_e\) | \(H_o\) (fixed alleles) | \(F_{IS}\) (loci deviated from HW equilibrium) | BayesAss migration rates |
|------------|---------------------|-----------------------------------|--------|-------------------------|-----------------------------------------------|-------------------------|
| 1 (NE)     | 20                  | 4.31 (1)                          | 0.63   | 0.31 (1)                | 0.1 (5)                                       | 0.17                    |
| 4 (NE)     | 20                  | 3.88                              | 0.55   | 0.32 (1)                | 0.19 (2)                                      | 0.27                    |
| 5 (NE)     | 20                  | 4.22 (1)                          | 0.61   | 0.33                    | 0.12 (3)                                      | 0.28                    |
| 9 (SW)     | 13                  | 4.69 (2)                          | 0.69   | 0.27                    | 0.48 (4)                                      | 0.31                    |
| 11 (SW)    | 19                  | 3.81                              | 0.60   | 0.37 (1)                | 0.05 (3)                                      | 0.18                    |
| 13 (SW)    | 20                  | 3.50                              | 0.57   | 0.43 (1)                | 0.03 (3)                                      | 0.18                    |
| 14 (SW)    | 18                  | 4.05                              | 0.63   | 0.40                    | 0.05 (0)                                      | 0.29                    |
| 15 (SW)    | 19                  | 3.69                              | 0.54   | 0.22 (1)                | 0.43 (3)                                      | 0.32                    |
| 16 (SW)    | 20                  | 4.17                              | 0.62   | 0.35 (1)                | 0.12 (3)                                      | 0.32                    |
| 21 (SW)    | 16                  | 4.43                              | 0.67   | 0.31                    | 0.22 (4)                                      | 0.22                    |
| 26 (SW)    | 19                  | 3.02 (1)                          | 0.50   | 0.30                    | 0.1 (2)                                       | 0.17                    |
| 27 (SW)    | 20                  | 4.59 (2)                          | 0.68   | 0.35                    | 0.3 (3)                                       | 0.32                    |
| 28 (NE)    | 20                  | 3.20                              | 0.46   | 0.24                    | 0.12 (1)                                      | 0.31                    |
| 31 (NE)    | 19                  | 3.73 (1)                          | 0.57   | 0.38                    | 0.05 (2)                                      | 0.32                    |
| 33 (NE)    | 20                  | 4.57 (1)                          | 0.67   | 0.29                    | 0.18 (4)                                      | 0.18                    |
| 35 (NE)    | 13                  | 4.17                              | 0.58   | 0.26 (1)                | 0.27 (2)                                      | 0.27                    |
| 37 (NE)    | 18                  | 3.61                              | 0.59   | 0.40                    | 0.08 (1)                                      | 0.08                    |
| 40 (NE)    | 20                  | 4.29 (1)                          | 0.62   | 0.38                    | 0.13 (5)                                      | 0.13                    |
| 42 (NE)    | 20                  | 4.74 (1)                          | 0.67   | 0.30 (1)                | 0.29 (3)                                      | 0.29                    |
| 47 (NE)    | 18                  | 3.84 (1)                          | 0.53   | 0.19 (1)                | 0.54 (5)                                      | 0.32                    |
| 49 (SW)    | 19                  | 3.93                              | 0.56   | 0.24 (1)                | 0.18 (4)                                      | 0.18                    |
| Mean ± SD  | 18                  | 4.02 ± 0.47                       | 0.60 ± 0.06 | 0.32 ± 0.06 | 0.2 ± 0.15 | 0.2 ± 0.15 |
step of 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 56 °C and 1 min at 72 °C, followed by a final extension step of 7 min at 72 °C. PCRs were performed in a 11000 thermocycler (Bio-Rad, CA, USA) and checked on agarose gels. Samples were run on an ABI 3730-XL Genetic Analyzer (Applied Biosystems, CA, USA) mixed with 9 µL HiDi Formamide and 0.1 µL Genescan 500 LIZ (Applied Biosystems) internal size standard. Fragment sizes were assigned to alleles using GeneMarker v1.75 (Softgenetics LLC, PA, USA).

Statistical analyses. Fragmentation effects: genetic diversity and correlations with patch characteristics

Expected and observed heterozygosity (\(H_e\), \(H_o\), respectively) for each fragment population were estimated using Genepop (Raymond and Rousset 1995). Micro-Checker 2.2.3 (Oosterhout et al. 2004) was also used to evaluate errors in genotyping and to detect the presence of null alleles. \(F_{st}\) was estimated using IN2est software (Chybicki and Burczyk 2009) that corrected for the excess of homozygosity due to the effects of null alleles and genotyping errors. The deviations from Hardy–Weinberg equilibrium and the presence of linkage disequilibrium were estimated using Genepop. To avoid the effect of differences in population size on the estimation of allelic richness, HP-RARE 1.0 (Kalinowski 2005) was used, using the minimum number of individuals sampled (i.e. 13 individuals in patches 9 and 35).

To evaluate the relationship between genetic parameters, patch characteristics and population sizes of \(H. squamatum\), all genetic parameters (i.e. rarefied allelic richness, average number of alleles, \(H_e\), \(H_o\), \(F_{st}\), migration rate from BayesAss) were tested for association with fragment variables (i.e. area, perimeter, population density) and connectivity values (C) employing Spearman’s correlation coefficient with R (R Core Team 2017, version 3.3). Soil patch characteristics (i.e. pH, nitrogen, phosphorus, etc.; see above) were tested for correlation with demographic parameters (and tentatively also with genetic variables, although no association was expected since microsatellites are expected to be neutral). Additionally, general linear models were implemented for \(H_e\), rarefied allelic richness and \(F_{st}\) as Gaussian dependent variables and patch area, connectivity and distance to the road as fixed factors using R (R Core Team 2017, version 3.3).

Population differentiation and genetic structure evaluating highway effects

\(F_{st}\) values between population pairs were estimated using FSTAT (Goudet 1995) with 10000 permutations. To evaluate the effects of the highway, we grouped the populations according to their position on either side of the highway (NE and SW) and compared the genetic parameters (i.e. allelic richness, \(H_e\), \(H_o\), \(F_{st}\) and \(F_{IS}\)) between both groups using FSTAT. We also used BayesAss software (Wilson and Rannala 2003) to estimate recent migration rates between populations/fragments since this approach is less influenced by deviations from Hardy–Weinberg equilibrium than others. Analysis was performed with 1 million burn-in generations, followed by 10 million interactions with a sampling frequency of 100. As we were more interested in knowing the migration rates from one fragment to the rest (instead of migration rates between two specific fragments), we calculated the sum of all migration rates that depart from each fragment.

Additionally, we calculated the average \(F_{st}\) values for all population pairs in each half of the landscape (NE and SW) and compared them to the average \(F_{st}\) values for all population pairs generated by a null model in which all populations from both sides of the highway were included. If the average \(F_{st}\) on one roadside was significantly lower than \(F_{st}\) values obtained from the null model, we could infer that the highway is acting as a barrier blocking gene flow between the patches situated on either side. To generate the null model we performed 1000 replications of the average of 100 \(F_{st}\) values randomly taken from all the available \(F_{st}\) values of all fragment population pairs. The \(F_{st}\) values obtained from each roadside were compared against the upper and lower 2.5 % \(F_{st}\) values of the distribution of the null model. The same approach was followed using migration rates estimated by BayesAss between populations instead of \(F_{st}\) values, assuming that these migration rates are more appropriate to test recent gene flow between patches.

To evaluate the presence of IBD, a Mantel test was carried out comparing spatial and genetic distance, using the Vegan package in R (Oksanen et al. 2007). We also used the spatial autocorrelation analysis implemented in SPAGeDi version 1.4 (Hardy and Vekemans 2002). This approach is based on genetic kinship coefficients between pairs of populations i and j (\(F_{ij}\)). \(F_{ij}\) were regressed on the spatial distance between populations and SEs were estimated by jackknifing over loci. For each distance interval, computed values were compared to a 95 % confidence interval (CI) around the null hypothesis (absence of IBD). The 95 % CI was constructed by performing 9999 random permutations of genotypes among spatial positions.

Finally, we carried out Bayesian clustering analyses to combine different statistical methods to detect critical fragmentation events (Kierepka and Latch 2015). We performed the analyses with InStruct, whose algorithm has been specifically designed for species with partial self-fertilization, allowing the software to estimate population-level
inbreeding coefficients (Gao et al. 2007). Based on an
Markov Chain Monte Carlo (MCMC) approach, five independ-
ent runs for each K value, ranging from 1 to 21, were per-
formed to determine the number of clusters, with 200 000
MCMC interactions after a burn-in period of 100 000. To eval-
uate the genetic structure with a non-Bayesian approach,
analyses of molecular variance (AMOVAs) were carried out
to quantify the proportion of molecular variance within and
among populations using GenAlEx 6.5 (Peakall and Smouse
2012). An AMOVA without prior assumption of population
structure was compared to another with a model that con-
sidered the population division due to the presence of the
highway (NE vs. SW populations).

**Results**

**Patch characterization, size, connectivity and
correlations**

The average rarefied allelic richness was 4.02 ± 0.47
(mean ± SD). Twelve private alleles were found in eight
fragments, while in 10 of these at least one mono-
morphic locus was found. Expected heterozygosity
ranged from 0.5 to 0.69 (0.6 ± 0.06) and observed het-
erozygosity ranged from 0.22 to 0.4 (0.32 ± 0.06). The
inbreeding coefficient, \(F_{IS}\), ranged from 0.05 to 0.48
(0.2 ± 0.15). Detailed values are depicted in Table 1. \(F_{ST}\)
values between population pairs were notably large
(0.24 ± 0.08) and ranged from 0.01 between fragment
populations 11 and 14 to 0.38 between populations 15
and 26 [see Supporting Information—Table S1]. The
sums of the migration rates for each fragment to the
others, estimated with BayesAss, also varied consider-
ably between populations (Table 1). Values of the sum of
migration rates ranged from 0.17 in patches 1 and 26 to
0.32 in patches 15, 16, 27 and 31, with an average ± SD
value of 0.25 ± 0.07.

Population density in each fragment varied greatly
(Table 2). The mean density (± SD) was 0.66 (± 0.76)
plants m\(^{-2}\), ranging from 0.01 to 3.05 plants m\(^{-2}\). The
mean value of the distance from the highway from the
centroid of each patch was 427 (± 306) m, whereas the

**Table 2.** Area (m\(^2\)) and perimeter (m) of each patch estimated from aerial photography. Density of *Helianthemum squamatum* in the sampled transects (plants m\(^{-2}\)), and connectivity index (\(C_i\)) (see Methods) are also shown.

| Population | Area (m\(^2\)) | Perimeter (m) | *H. squamatum* density | \(C_i\) |
|------------|---------------|---------------|------------------------|--------|
| 1          | 1269983       | 471           | 1.26                   | 3.44   |
| 4          | 4429          | 660           | 0.01                   | 1.00   |
| 5          | 3170          | 313           | 0.034                  | 2.73   |
| 9          | 16056         | 709           | 0.032                  | 1.96   |
| 11         | 20210         | 1006          | 1.64                   | 2.74   |
| 13         | 1019191       | 14786         | 3.05                   | 4.13   |
| 14         | 998043        | 9227          | 0.19                   | 4.10   |
| 15         | 1572          | 167           | 1.12                   | 3.75   |
| 16         | 4855          | 543           | 1.09                   | 3.19   |
| 21         | 8838          | 1186          | 0.07                   | 2.69   |
| 26         | 474           | 130           | 0.13                   | 3.14   |
| 27         | 2045          | 295           | 0.3                    | 4.20   |
| 28         | 13069         | 1059          | 0.26                   | 1.25   |
| 31         | 13670         | 859           | 0.54                   | 1.31   |
| 33         | 7639          | 599           | 0.24                   | 1.36   |
| 35         | 1901          | 369           | 0.01                   | 1.12   |
| 37         | 13546         | 674           | 0.35                   | 1.57   |
| 40         | 4615          | 521           | 0.34                   | 1.46   |
| 42         | 2224          | 239           | 0.41                   | 1.34   |
| 47         | 1013167       | 16735         | 1.61                   | 4.15   |
| 49         | 998043        | 9227          | 1.06                   | 4.10   |
| Mean ± SD  | 198069 ± 402192 | 2846 ± 5030 | 0.66 ± 0.76            | 2.61 ± 1.19 |
distance from the closest border of the patch was 328 (± 269) m. Similar to the genetic coefficients, the values of the connectivity index showed high variation. The mean value was 2.61 ± 1.19, ranging from 4.19 (fragment 27) to 0.99 (fragment 4). Soil characterization values of each patch are shown in Supporting Information—Table S2.

Genetic parameters (except $F_{ST}$) showed no correlation with population density or distance from the highway. $F_{IS}$ showed a negative correlation with population density ($\rho = -0.43$, $P$-value = 0.04). None of the estimated genetic values was correlated with patch area or fragment perimeter (Table 3). Non-significant differences in $H_E$, $H_O$, $F_{IS}$ and $F_{ST}$ were found between the two groups of populations located on either side of the highway (Table 4). Population density was not correlated with landscape variables or with soil estimates. Generalized linear models found no significant associations between $H_E$, rarefied allelic richness or $F_{IS}$ and patch area, connectivity and distance to the road [see Supporting Information—Table S3].

**Highway effects in population structure**

None of the analyses performed showed a relationship between genetic parameters and fragmentation structure, suggesting the absence of an IBD pattern. The Mantel test showed non-significant results ($r = 0.1$, $P = 0.15$) and no spatial autocorrelation was found by the SPAGeDi analysis [see Supporting Information—Fig. S1]. Bayesian clustering analyses suggested a vague genetic structure formed by 14 clusters (Fig. 2), without a clear spatial pattern. Analyses of molecular variance results showed the same significant distribution of molecular variance in both models (with highway partition and without). Most molecular variation was included within populations (80%).

The comparisons against a null model of complete randomness showed that the average pairwise $F_{ST}$ values of the SW populations, considered separately, were not significantly different than pairwise $F_{ST}$ values of all the 21 populations from both sides of the highway. On the contrary, the average $F_{ST}$ value of the NE populations ($F_{ST} = 0.22$) was significantly higher than the 97.5 % random values generated by the null model ($F_{ST} = 0.19$), suggesting that the differentiation between the fragments on this side is higher than random expectations. The comparisons of the migration rates, calculated with BayesAss against a null model of complete randomness, showed similar patterns. No significant differences were found in the migration rates between the SW populations (0.125) and the random average generated (0.125) while the average migration rates between the NE populations was lower (0.109).

**Discussion**

As hypothesized, our results show that the interaction of several fragmentation mechanisms has a weak effect in this pervasive gypsum soil specialist. This concurs with our idea that gypsophytes have evolved in island-like scenarios with high isolation between patches (Sanchez et al. 2017). This adaptation may hamper the emergence

| Allelic richness rarefied | Average number of alleles | $H_E$ | $H_O$ | $F_{IS}$ | BayesAss migration rate |
|--------------------------|--------------------------|-------|-------|--------|-------------------------|
| Patch area (m)           | -0.16                    | -0.2  | -0.03 | 0.22   | 0.12                    |
| Patch perimeter (m)      | -0.21                    | 0.27  | -0.13 | 0.14   | 0                       |
| $H$. squamatum density (plants m⁻²) | -0.3                   | -0.06 | -0.19 | 0.11   | -0.27*                  |
| $C_i$ (patch connectivity) | -0.11                    | 0.12  | 0.05  | 0.08   | -0.02                   |
| Organic C                | -0.11                    | 0.09  | -0.04 | 0.23   | -0.13                   |
| Soil conductivity        | -0.11                    | -0.06 | -0.18 | -0.17  | 0.01                    |
| Nitrogen                 | -0.37                    | -0.13 | -0.13 | 0.09   | -0.05                   |
| Potassium                | -0.2                     | 0.07  | 0.01  | 0.17   | 0.21                    |
| Phosphorus               | 0.06                     | 0.14  | 0.2   | 0.23   | -0.08                   |
| pH                       | 0.45*                    | 0.32  | 0.51* | 0.05   | 0.04                    |
| Glycosidase              | -0.21                    | -0.04 | -0.02 | 0.09   | 0.07                    |
| Phosphatase              | -0.29                    | -0.36 | -0.24 | -0.17  | -0.22                   |

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**Table 3.** Summary of correlation (Spearman’s $R$) values between genetic parameters and landscape, soil and population size values in each fragment. * refers to values with significant correlation ($P$-value < 0.05). Glycosidase and phosphatase activity were measured in μmol/(g·h)⁻¹; nitrogen in mgN g⁻¹ soil; P in mgP g⁻¹ soil; K in mgK g⁻¹ soil; conductivity in µS cm⁻¹ and organic C in %.
of pernicious fragmentation effects even when natural insularity is exacerbated by different human-driven drivers (Escudero et al. 2015; Harrison et al. 2015; Matesanz et al. 2017).

*Helianthemum squamatum* genetic diversity and selfing coefficients varied considerably among the populations of each gypsum habitat remnant. As hypothesized and contrarily to findings in other gypsum specialist plant species (Gómez-Fernández et al. 2016), none of the genetic parameters were associated with the spatial descriptors of the landscape structure (i.e. fragment size and connectivity) or to the existence of an IBD pattern at wider scales (Martínez-Nieto et al. 2013). In addition, variation in the genetic parameters was not related to demographic estimates, such as variations in density, or to soil parameters. Combining these results, it seems evident that the genetic structure of the metapopulation is not significantly influenced by landscape configuration (fragmentation and infrastructure). The intense fragmentation scenario due to agriculture, natural gypsum habitat configuration and the presence of human infrastructure, limiting gene flow between habitat remnants, is somehow counteracted by the biological features of the species, such as specific species traits (seeds with mucilage coats, Escudero et al. 1999; general pollinators, Aragón and Escudero 2008; plastic responses, Sanchez et al. 2017), the evolutionary processes of the species and the historical context of the habitat.

Genetic diversity values and structure in *H. squamatum* are congruent with the biological features of the species, including a mixed-mating system (Aragón and Escudero 2008), the presence of a long-standing soil seed bank (Caballero et al. 2008), and the absence of specialized characters modulating seed dispersal including myxospermy, which promotes anchorage of seeds in the close vicinity of mother plants, thereby limiting dispersal (Gutterman 1993). These features may be responsible for the low gene flow found, the existence of monomorphic loci in some patches and the presence of moderate to high levels of *Fst*. The low gene flow values and the presence of moderate levels of inbreeding in some populations may not necessarily infer a survival disadvantage for the populations, as found in other widely distributed gypsum specialists (Martínez-Nieto et al. 2013; Gómez-Fernández et al. 2016). Only when fragmentation and disturbance reduced the size of the fragment to a point at which the population density of *H. squamatum* dropped significantly, inbreeding depression effects may appear compromising plant fitness and population viability.

The paradox of a widespread species with biological mechanisms promoting restricted gene flow and, of greater importance, genetic differentiation among fragments not related with landscape configuration needs additional considerations. Some of the biological features that could help to solve this apparent contradiction are related with the high reproductive rate (Aragón and Escudero 2008) and a low life expectancy of *H. squamatum* coupled with highly dynamic and fluctuating populations (Aragón et al. 2009; Tye et al. 2017). The self-pollinating capability of the species, along with the generalist nature of its pollinators, allows successful reproduction even under less favourable environmental conditions (Escudero et al. 1999; Aragón and Escudero 2008). The presence of seed dormancy provided by the hard-coated seeds allows their persistence in the soil bank and fosters extraordinary recruitment events when favourable combinations of temperature and precipitation regimes occur (Escudero et al. 1999; Caballero et al. 2005; Aragón et al. 2009). Sporadic migration events through seed dispersal cannot be totally discarded. The presence of domestic livestock that are efficient dispersers in these anthropogenic landscapes (e.g. Manzano and Malo 2006) can modify the migration rates between patches, promoting occasionally LDD events. Furthermore, the pollination of *H. squamatum* by generalist species also facilitates a certain level of pollen

![Instruct clustering output. Each colour represents one of the 14 clusters proposed by the software. The numbers below the graph represent the studied fragments.](https://academic.oup.com/aobpla/article-abstract/10/1/plx072/4718095/4718095)
dispersal (Santamaría et al. 2017). In synthesis, some mechanisms favour recruitment in the mother source origin, whereas others related to the high fertility, existence of a long-standing soil seed bank, sporadic LDD and rapid population dynamics could explain the species widespread distribution and this apparent paradox.

The lack of an IBD pattern and a clear genetic structure may be associated with several factors, some of them acting in opposite directions (Ewers and Didham 2006). On the one hand, since most of the scarce gene flow is achieved through pollination (as seed dispersal is very inefficient), pollinator movement between fragments must be constrained by factors other than simple geographical distances (Ewers and Didham 2006; Santamaría et al. 2017). As pollination is performed by generalist pollinators, the flower display provided at a given time by the whole plant community in all the fragments is likely to determine the attraction to each site. On the other hand, simulations suggest that past landscape configuration (with more and larger fragments and/or a matrix less aggressive than current conditions) and the simultaneous presence of several generations of individuals in the same fragment (Olano et al. 2011) may also influence the current spatial-genetic structure of the landscape (Landguth et al. 2010). However, the highly dynamic demographic patterns of H. squamatum populations (Escudero et al. 1999; Olano et al. 2011) would complicate the existence of any delay in the genetic responses to current fragmentation.

Absence of highway effects on H. squamatum populations

It is noteworthy that, in spite of the different analyses performed, there is an absence of effects associated with the presence of the highway on the genetic diversity and structure of H. squamatum populations. Many theoretical studies have evaluated the effects of barriers in a landscape and the factors that modulate its impact using genetics approaches. These computational models suggested that several factors such as the population’s demography or species’ biology (especially pollination and seed dispersal features) are essential for detecting barrier or corridor effects (Landguth et al. 2010; Cushman et al. 2012) over the distribution of genetic diversity of a species in an area. The life history and population dynamics of H. squamatum may delay the appearance of fragmentation effects in gene diversity patterns. However, the genetic consequences already manifested in small habitat patches may become widespread if the available habitat keeps decreasing in the gypsum landscapes. Finally, the scarce number of empirical studies that so far have been carried out also highlight the complexity of detecting genetic effects of roads in natural plant populations (see Holderegger and Di Giulio 2010), but are essential to improve the design of future infrastructures (Karlson et al. 2016).

Conclusions

Biological features arising from evolution on natural edaphic islands and population dynamics seem to buffer the effects of human-induced fragmentation over H. squamatum populations. Under these premises, results suggest that the preservation of fragments of large size and high population density should be a priority for the conservation of endangered gypsum communities, in order to maintain genetic diversity and avoid the pernicious effects of genetic depauperation.

Sources of Funding

This work has been partially supported by the Spanish Ministry of Economy and Competitiveness through the ROOTS (CGL2015-66809), ECOMETAS (CGL2014-53840-REDT) and EVA (CGL2016-77377-R) projects. Project REMEDINAL-3 (S2013/MAE-2719) also provides funding for this study.

Contributions by the Authors

A.G.F.: study design, perform lab and field work, analyse results and manuscript writing. A.E.: study design, analyse results and manuscript writing. J.M.I.: study design, analyse results and manuscript writing. B.H.R.: perform lab and field work, analyse results and manuscript writing.

Conflict of Interest

None declared.

Acknowledgements

Authors are indebted to C. Diaz for his help during field surveys and I. Mola for his assistance during the project.

Supporting Information

The following additional information is available in the online version of this article—

Table S1. F_ST values between population pairs.

Table S2. Soil characterization of the fragments studied.

Table S3. General linear models of genetic diversity (H_E, rarefied allelic richness, FST) and patch variables.

Figure S1. Spatial autocorrelation calculated with SPAGeDi.
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