# Genetic diversity and structure of the narrow endemic species *Crepis granatensis*: implications for conservation

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## Abstract

In this study, we studied the genetic diversity and population genetic structure of the endangered endemic *Crepis granatensis*, using amplified fragments length polymorphism (AFLP) and plastid DNA (cpDNA). No genetic divergences were obtained using cpDNA markers. Three primers combinations selected from a total of 12 produced a total of 421 fragments, of which 418 (99.3%) were polymorphic. The total genetic diversity of *C. granatensis* was moderate (Ht = 0.260). Nei´s gene diversity ranged from 0.202 to 0.258. The fixation index (Fst) was 0.137, suggesting low to moderate genetic differentiation among populations. The AMOVA analysis revealed that genetic diversity was mainly concentrated among individuals within populations (74%), while 8% was found among populations and 18% among regions. The Bayesian analysis and PCoA identified two genetic clusters: one corresponded to La Sagra population and the other corresponded to the Mágina populations. Based on our genetic results, it is necessary to preserve the evolutionary potential of *C. granatensis* by protecting all extant populations. Both *in situ* and *ex-situ* conservation measures should be considered. Reinforcement, reintroduction, and translocation programmes could be performed if necessary. Finally, such conservation strategies should be considered both in the current recovery plan and management actions for the species.



**Keywords**: AFLPs; conservation genetics; cpDNA; mountain plants; naturally rare species; screes species.

## Introduction

Conservation of genetic diversity is one of the main goals of biodiversity conservation (Gordon et al. 2012) since it influences the species genetic patterns and their adaptability, survival, and reproduction in rapidly changing habitats or newly colonized habitats. In particular, rare and threatened species with narrow geographical distribution (narrow endemics) have a greater risk of decline and extinction than widespread species since they are characterized by having a reduced distribution range, fragmented populations, small population size, and long-term isolation. In addition, on many occasions, they grow in specialized habitats with specific requirements (Levin 2019). The causes of species rarity can be a combination of genetic, ecological, and historical factors (Kruckeberg and Rabinowitz 1985). The fact that 60% of the endemic plant species of the Mediterranean region are narrow endemics (Thompson 2005), they have received little attention compared with widespread species.

A species becomes a rare species due to human disturbances (new rare species) or by being associated with specific habitats and particular geographical locations (naturally rare species or old rare species). The latter show a naturally fragmented distribution and geographically isolated populations which lead to a decrease of gene flow and genetic diversity and increase genetic drift and genetic differentiation among populations (Cole 2003; Frankham et al. 2010; Rodríguez-Peña et al. 2014). However, in some cases, this rule of thumb does not apply; thus other rare species show high genetic diversity and low differentiation among populations (e.g. *Aster pyrenaeus* DC. (Escaravage et al. 2011), *Campanula sabatia* De Not (Nicoletti et al. 2012), *Pseudomisopates rivas-martinezii* (Sánchez Mata) Güemes (Jiménez Mejías et al. 2015), indicating that they are well adapted to such a spatial distribution.

Screes are considered as specific habitats, characterized by having low vegetal coverage and diversity but high levels of specialized endemic species. Most of these are classified as Endangered and listed in some Red List and/or Regional catalogues. Regardless of the importance of screes species for conservation, so far not much attention has been paid to them. As model species for this study, we have chosen *Crepis granatensis* (Willk.) Blanca & Cueto (Asteraceae) (for the description, see Blanca and Cueto 1985), a naturally narrow endemic of the south-eastern mountain ranges of the Iberian Peninsula, in particular Sierra Mágina in Jaen Province and Sierra La Sagra in Granada Province. Although it was also reported in Sierra de Gádor (Almería province) and Sierras Cazorla-Segura (Jaén province), it has not been recently found. It grows in calcareous screes of high mountains with strong slopes, where the movements of rocks are frequent. The high specialization and low ecological plasticity lead to a naturally fragmented distribution. The number of individuals per population is highly variable, but only one population exceeds 2000 individuals (Blanca et al. 2003; Melendo et al. 2011). It is included in the association *Crepido granatensis-Iberidetum granatensis* Quézel 1953 (*Platycapno saxicolae-Iberidion lagascanae* Rivas Goday & Rivas-Martínez 1963; *Thlaspietalia rotundifolii* Br.-Bl. 1926) (Blanca et al. 1987). It coexists with other screes species such as *Jurinea fontqueri* Cuatrec., *Vicia glauca* subsp. *giennensis* (Cuatrec.) Blanca & F. Valle, *Platycapnos saxicola* Willk. and *Lactuca perennis* subsp. *granatensis* Charpin & Fern. Casas. Its main pollinators are Hymenoptera and Lepidoptera, and seed dispersal is through the wind (anemochory) (Blanca et al. 2003). Its chromosome number is $2n = 8$ (Blanca and Cueto 1985). The main threats are caused both by natural risk factors (high specificity of habitat, low ecological plasticity, reproductive barriers, high percentage of non-germinated seeds, low rate of survival of seedlings) and by anthropogenic risk factors (excess of livestock

and mountain sports) (Blanca et al. 2003). The species is listed at different levels: International level (IUCN (Blanca et al. 2013) as EN; European level (Annexes I and V of the Bern Convention and Annex II of the Directive Habitat); national level (Red List of the Spanish Vascular Flora 2008 (Moreno 2008) as EN (B1ab(iii,v) + 2ab(iii,v); and at Regional level (Andalusian Catalogue of Threatened Species (BOJA 2012a) as Endangered). A recovery plan was also implemented in Andalusia (BOJA 2012b).

Studies of genetic diversity of rare and threatened plants –for example, narrow endemics– provide relevant information on population dynamics, evolutionary relationships, levels of genetic diversity and within- and among-population genetic structure, evolutionary processes (i.e. loss of diversity, genetic drift and bottlenecks) (Gitzendanner and Soltis 2000; Frankham 2005). Likewise, such studies clarify not only the reproductive strategies but also provide useful information for management and biological conservation (Frankham et al. 2010; Lopes et al. 2014). As yet, genetic studies on rare and endemic screes species are scarce in the Iberian Peninsula.

Thus, molecular markers are useful tools frequently used in plant genetic diversity. The choice of type of marker used could affect inferences of population genetic parameters. In this study, we selected two types of markers: amplified fragment length polymorphism (AFLP) and plastid DNA (cpDNA). AFLPs (Vos et al. 1995) are a common, reliable and replicable DNA fingerprint method that has been successfully applied in surveying the population genetic structure and genetic diversity in plant conservation studies (Wang et al. 2012). Plastid DNA is a molecular marker widely used for taxonomy, plant phylogeography and evolutionary research (Taberlet et al. 1991; Avise 2009) and characterized by its maternal inheritance, absence of recombination and high level of genetic diversity (Wheeler et al. 2014).

In this study, we assessed the genetic characterization of *C. granatensis* for its whole distribution range, using AFLP and cpDNA markers. No information on any genetic aspects of this species is available so far. The aims of this study were to: (1) identify the level of genetic diversity of *C. granatensis*; (2) quantify how the genetic variability is distributed within and among populations; and (3) propose useful strategies for conservation, management and restoration, based on our genetic results, for this endangered species.

## Materials and methods

### *Population sampling*

Plant material was collected covering the whole distribution range of *C. granatensis* (Fig.1). We sampled a total of 100 individuals, ranging from 18 to 33 per site, from four populations: one population corresponding to Sierra de La Sagra in Granada Province (SAGRA) and three populations to Sierra Mágina in Jaén Province (MAG1, MAG2, MAG3) (Figure 1; Table 1). To avoid DNA degradation, sampling material –one or two leaves per plant- was bagged in plastic bags with silica-gel until DNA extraction (Chase and Hills 1991; Sytsma et al. 1993).

### *DNA extraction and AFLP analysis*

DNA was extracted from silica gel dried leaves using the 2 × cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). Total DNA extracts were quantified using a Nanodrop 2000 spectrophotometer. Amplified fragment length polymorphism (AFLP) analysis was carried out following Vos et al. (1995) with minor modifications as follows. The analysis was performed with fluorescence-labeled primers (FAM, VIC, PET, Applied Biosystems, Madrid, Spain) instead of radioactively labeled primers. Fragments were selectively amplified with the primer pairs EcoR1- ATG/MseI-CGT,

EcoRI-ACA/MseI-CGT, EcoRI-ACG/MseI-CAC. Multiplex products were run for 4 h on an ABI 377 sequencer to separate fragments together with an internal size standard (GeneScan 600 LIZ, ABI). Sizing and peak identification were performed using Genemapper 4.0 software (Applied Biosystems). In the finally assembled binary matrix, the presence of a band was scored as 1 while the absence of a band was scored as 0. Furthermore, several cpDNA regions (trnL-trnF spacer, trnH-psbA intergenic spacer, trnS-trnG intergenic spacer), were sequenced in 12 individuals (three individuals from each population), to explore geographic variability. Amplification reactions were conducted in 50 µl volumes containing approximately 20 ng of genomic DNA, 0.2 mM of each dNTP, 2.5 mM MgCl2, 2 units of Taq Polymerase (Biotools), the buffer provided by the manufacturer and the primer combinations trnL-trnF for trnL-trnF intergenic spacer (Taberlet et al. 1991), trnH (GUG)-psbA for trnH-psbA intergenic spacer and trnS (GCU)-trnG (UCC) for trnS-trnG intergenic spacer (Hamilton 1999), at a final concentration of 0.4 mM. Reactions were performed in an Eppendorf Mastercycler using the following program: an initial cycle at 94ºC for 3 min; 35 cycles of 30 s at 94ºC, 30 s at 52ºC, and 1 min at 72ºC. A final cycle at 72ºC for 8 min was included to terminate amplification products. Finally, 2 µl of the amplification products were visualized on 1.5% agarose gel and successful amplifications were cleaned with the GenElute PCR clean-up kit (Sigma-Aldrich, Madrid). For sequencing, purified PCR products were reacted with BigDye terminator cycle sequencing ready reaction (Perkin-Elmer, Applied Biosystems, Madrid) using amplification primers. For each product, both strands were sequenced. Unfortunately, neither interpopulation nor intrapopulation variation was detected (data not shown).

***Data analysis***

As AFLP markers are dominant, we assumed that null bands were homologous and that populations were in Hardy-Weinberg equilibrium (Lynch and Milligan 1994) to compute diversity indices [percentage of polymorphic markers (PLP) and Nei's (1978) unbiased expected heterozygosity (He)] and genetic distance among populations (Fst). These parameters were inferred with AFLP-surv 1.0 (Vekemans 2002). Significance of Fst values was determined using 1000 bootstrapped data sets. We calculated the frequency-down-weighted marker (DW value) (Schönswetter and Tribsch 2005), a standardized measure of divergence which estimates the genetic rarity of a population as equivalent to range down-weighted species values in historical biogeographical research (Crisp et al. 2001). For each population, the number of occurrences of each AFLP marker in that population was divided by the number of occurrences of that particular marker in the total dataset. Finally, these values were summed up. The value of DW is expected to be high in long-term isolated populations where rare markers should accumulate due to mutations whereas newly established populations are expected to exhibit low values, thus helping in distinguishing old vicariance from recent dispersal. DW parameter (frequency-down-weighted marker values) was calculated using the R package AFLPdat (Ehrich 2006). Genetic structure analysis was performed using analysis of molecular variance (AMOVA) to estimate components of variance partitioned within and among populations (Excoffier et al. 1992). The program ARLEQUIN v.3.5. (Excoffier and Lischer 2009) was used for performing this test, with significance test by 10000 permutations.

A Principal Coordinate Analyses (PCoA) was performed to illustrate overall similarity among individuals using Genalex 6.5. PCoA was inferred from the pairwise Nei's genetic distance (Nei 1978) between all pairs of AFLP phenotypes.

Pairwise Fst-values between all populations were also calculated and tested for significance by resampling with 1000 random permutations using AFLP-surv 1.0 (Vekemans 2002). Pairwise gene flow (Nm) values between populations were estimated based on Fst using the formula Nm = [(1/Fst)-1]/4) (Slatkin and Barton 1989). Mantel tests (Mantel 1967), were performed to assess linear correlation among genetic and geographic distances using Genalex 6.5 (Peakall and Smouse 2006).

A Bayesian model-based analysis was performed to infer population structure with Structure version 2.3 (Pritchard et al. 2000; Falush et al. 2007). The F model, based on an admixture ancestry model with correlated allele frequencies, was imposed to estimate the posterior probabilities [LnP(D)] of K groups (Pritchard and Wen 2004) and the individual percentages of membership assigned to them according to their molecular multilocus profiles (Falush et al. 2003, 2007). Probabilities for a range of K were examined starting from one to the number of sampled populations plus one (K = 1-5), using a burn-in period and run length of the Markov chain Monte Carlo (MCMC) of 105 and 106 iterations, respectively, replicated 20 times. Results were uploaded into STRUCTURE HARVESTER (Earl and von Holdt 2012, available at http://taylor0.biology.ucla.edu/struct_harvest/), which estimates the most likely K value (ΔK), following Evanno et al. (2005). We used CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) to reach a consensus on the results of the independent runs for the optimal K. For the consensus, we used the Greedy option with random input order and 10000 repeats. The consensus was visualized in DISTRUCT 1.1 (Rosenberg 2004).

**Results**

Alignment of the 12 individuals for each cpDNA region yielded sequences reaching 872 nucleotides for trnL-trnF spacer, 389 for trnH-psbA and 692 bp for trnG-trnS spacer

(data not shown). Unfortunately, all the individuals shared identical sequences, so no genetic divergences were obtained using cpDNA markers. The three selective AFLP primer combinations amplified 421 reproducible fragments, of which 418 were polymorphic (99.3%). The first (EcoR1-ATG/Msel-CGT) gave 139 fragments (33.02%) between 65 and 308 base pairs (bps), the second (EcoRI-ACA/Msel-CGT), 116 fragments (27.55%) between 71 and 265 bps and the third (EcoRI-ACG/Msel-CAC), 166 fragments (39.43%) between 62 and 327 bps. All the 100 individuals had unique AFLP profiles. No private markers for population were detected, but we found 2 exclusive bands for La Sagra population, which appeared in more than 50% of sampled individuals, and 4 exclusive bands for Mágina populations. The percentage of polymorphic loci (PLP) for a single population ranged from 58.4% (MAG2) to 77.4% (SAGRA) (Table 1). Expected heterozygosity values (or Nei's gene diversity, Hj) showed that SAGRA was the genetically most variable population (Hj = 0.258), whereas MAG2 showed the lowest within-population genetic diversity (Hj = 0.202). The average gene diversity within populations (Hw) was 0.225, and the total genetic diversity (Ht) was 0.260 (Table 1), indicating a moderate level of genetic diversity in *C. granatensis.* SAGRA population showed the highest value of frequency down-weighted marker values (DW) while MAG2 exhibited the lowest value (Table 1).

The fixation index was highly significant (Fst = 0.137, P < 0.000), suggesting low to moderate genetic differentiation among populations. Analysis of Molecular Variance (AMOVA) displayed that the overall differentiation was low. Most of the total genetic variation was concentrated within populations (74%), whereas only 8% was distributed among populations (Table 2). The remaining 18% was explained by differences between the two study regions (La Sagra-Mágina).

The Bayesian analysis of the genetic structure of *C. granatensis* populations conducted with Structure found the highest estimate of the likelihood of the data (LnP(D)) and ΔK values for K = 2 (Figure 2). SAGRA population was assigned to one cluster and Mágina populations were included in the second cluster. Some individuals showed a proportion of membership intermediate between these two clusters in every population (Figure 2). PCoA analyses gave similar results (Figure 3). PCoA plot revealed a clear separation between La Sagra and Mágina populations, although some individuals from MAG2 population were located near individuals from SAGRA population in the multivariate space, indicating weak differentiation. For this analysis, the three first axes accounted for 31% of the variation (15.25%, 9.88% and 5.87%, respectively).

Pairwise Fst between populations showed moderate to high differentiation between La Sagra and Mágina populations. In contrast, very low pairwise Fst was observed between Mágina populations (Table 3). Pairwise gene flow (Nm) values between populations are in line with the previous results, ranging from 0.967 to 5.908, being the lowest value between MAG1-SAGRA and the highest between MAG3-MAG1 (Table 4). The Mantel test displayed a high value of R but no significant correlation between genetic and geographical distances (R = 0.918, P = 0.224).

## Discussion

### *Genetic diversity and structure*

Narrow endemic species typically show lower genetic diversity and higher genetic variability than widespread species (Hamrick and Godt, 1996) due probably to their peculiar features (see Levin 2019). However, there are some exceptions of narrow endemics species, in which genetic diversity values are similar to widespread species

(Gitzendanner and Soltis 2000; Cole 2003). One of such exceptions is *C. granatensis* which shows moderate levels of total genetic diversity (Ht = 0.260). This fact can be accounted for by a recent decline in population size, short isolation period and regular gene flow (Chiang et al. 2006). The comparison of genetic values between studies is generally not advisable since many factors affect levels of genetic diversity (e.g., historical processes and evolutionary history, life traits, life-forms, geographical distribution range, population size, and type of molecular marker used). Regardless of these issues, the total genetic diversity obtained for *C. granatensis* is higher than the mean value for angiosperms (Ht = 0.221) (Nybom 2004). Furthermore, it can be compared with those obtained using AFLPs for other Iberian narrow endemic species (Fernández-Mazuecos et al. 2014; Jiménez-Mejías et al. 2015; Forrest et al. 2017).

Many factors might determine genetic diversity, being more significant extrinsic historical factors than intrinsic factors, among them genetic composition and any life-trait of the species (Jiménez-Mejías et al. 2015). In particular, the breeding system affects the current levels of genetic diversity and structure of the vascular plant populations (Hamrick and Godt 1996). Pollination of *C. granatensis* is entomophilous, being Hymenoptera and Lepidoptera the most common groups of pollinators (Blanca et al. 2013). Although there is no available experimental survey about the breeding system of *C. granatensis*, we assumed, from the genetic pattern obtained and the field data observations (Blanca et al. 2003), that it is an outcrossing species.

The AMOVA analysis showed that most of the genetic variation was assigned among individuals within populations (74%), with only 8% concentrated among populations. This genetic structure has been reported for other narrow endemic species (García-Fernández et al. 2013; Cánovas et al. 2015; Jiménez et al. 2017), being well-known in long-lived and outcrossing plants (Hamrick and Godt 1996), whereas the

opposite pattern occurs in selfing and mixed and annual plants (Nybom 2004). Outcrossing guarantees pollen dispersal, at least, among individuals of the same population or nearby populations –e.g., Mágina populations–, ensuring gene flow, maintaining genetic diversity and evolutionary potential of *C. granatensis*, reducing thus the probability of extinction (Frankham 2005).

The genetic variation pattern exhibited for *C. granatensis*, that is, lower concentration among populations and higher within populations suggested that the isolation, and subsequent differentiation of populations, occurred recently. This hypothesis is supported by cpDNA results. The absence of genetic divergences found using cpDNA markers could have two possible explanations: (1) either the populations have separated in relatively recent times, without enough time to generate mutations in the studied regions, or (2) recent long-distance gene flow has masked the possible differentiation at the plastidial level. The speciation process of *C. granatensis* was likely relatively recent, as similarly occurred in other Iberian endemic groups such as *Delphinium* L. ser. *Fissa* Pawl. (Ramírez-Rodríguez et al. 2019) and *Dianthus pungens* L. gr. (Castro et al. 2020).

Two different clusters were differentiated: one group corresponds to SAGRA population and the second group includes the Mágina populations. According to the Fst and Nm values, the genetic divergence among populations was high between regions and low between Mágina populations. The MAG2 population has the lowest Fst and DW values (Hj = 0.202; DW = 85.1), which suggests that this population may be either the most recent or the most affected or both. There is no doubt that it has suffered important pressures in the last decades due mainly to an excess of herbivores, which would affect the habitat quality and, as a consequence, the diminution in the number of individuals (Melendo, pers. obs.). Surprisingly, the PCoA analysis and the pairwise Fst

and Nm estimates suggest that the most geographically distant populations (MAG2 and SAGRA), separated by 87.67 km, have certain gene flow and similarities with each other. Both genetic differentiation among populations and between regions were highly significant ($P < 0.001$), which would be interpreted as isolation by distance. However, the Mantel test failed to reveal a correlation between geographical and genetic distances. According to these results, geographical distance does not affect gene flow between populations, which depends on pollen and seed dispersal (Petit et al. 1993). The gene flow (Nm) values found between Mágina populations reveal short-distance pollen dispersal by insects (entomophilous pollination). Although there are no data on pollen dispersal distances and insect pollinators, long-distance pollen dispersal events may sporadically occur between regions, which would explain the genetic relation between some individuals of MAG2 and SAGRA populations. The intermediate position of the recently extinct population in Sierra Cazorla (Blanca et al. 1987, 2003) may have played an important role in interconnecting Sierra Mágina and Sierra Sagra through pollen exchange. Regarding seed dispersal of *C. granatensis*, fruits are achenes with thistledown that disperse through the wind (anemochory). However, long-distance seed dispersal events seem unlikely to occur since both regions are located in complex geomorphology separated by approximately 85 km. Also, fruits and seeds, despite having adapted structures for wind dispersal, are not able to disperse over long distances since all fruits formed in a capitulum are united and intertwined by their thistledown, limiting dispersion as diaspores roll down through scree and soon find a hole between the stones (Melendo, pers. obs.). Nonetheless, the probability of seeds to reach, germinate and survive in a new suitable area is low due to the specificity of habitat (low ecological plasticity), low germination rate and low seedling survival, respectively (Blanca et al. 2013).

***Implications for conservation***

*Crepis granatensis* is a narrow endemic species with a reduced and severely fragmented distribution range as well as a relatively small population size, listed as EN (Endangered). This may lead to a loss of genetic diversity due to genetic drift and inbreeding depression (Young et al. 1996; Frankham et al. 2010). Nevertheless, and according to our results, gene flow (Nm) estimates among *C. granatensis* populations were, except for MAG1-SAGRA, relatively higher between Sagra and Mágina populations (and considerably higher between Mágina populations), than Nm = 1, the threshold value above which gene flow may consider significant. Therefore, populations may prevent significant genetic differentiation caused by genetic drift (Wright 1951; Slatkin 1987). Likewise, the levels of total genetic diversity and within-population genetic were moderate what means that the species is not genetically impoverished. As such, other factors –ecological and biological- different from genetic factors may account for the rarity of the species, such as low ecological plasticity (specificity of habitat), low germination rate, reduced seedling survival, and habitat heterogeneity (Melendo et al. 2011; Blanca et al. 2013). Consequently, to preserve as much genetic diversity as possible in extant populations and their evolutionary potential, both *in-situ* and *ex-situ* conservation measures should be implemented as well as reinforcement, reintroduction and translocation programmes, if necessary.

As far as in-situ conservation measures, the following interventions should be taken into account: (1) avoid the movements of rocks caused by mountain activities and herbivores, which damage plants, forbidding the access installing metal fences; (2) herbivory monitoring since herbivores may affect *Crepis granatensis* populations, specially MAG2 population, of two different ways. Firstly, in a direct way, herbivores eat and trample on them; secondly, herbivores, indirectly, caused habitat nitrification

through depositions, reducing habitat quality; (3) continue with the demographic, reproductive biology and ecological studies; (4) assess if the enclave of Sierra La Sagra should include in any defined protected area other than Special Conservation Zone (ZEC) Sierras del Nordeste (ES6140005); and (5) proposal to create, at least, two Plant Micro-Reserves (PMRs) in areas with high ecological, biological and conservation values. Consequently, one PMR should be created in Cárceles (Sierra Mágina) (MAG3 population) where *C. granatensis* coexist with *Jurinea fontqueri*, *Vicia glauca* subsp. *giennensis*, *Platycapnos saxicola* and *Galium rosellum* (Boiss.) Boiss. & Reut.) and another in Sierra La Sagra (SAGRA population) where it lives together with *P. saxicola*, *Lactuca perennis* subsp. *granatensis*, *Andryala agardhii* DC., *Senecio quinqueradiatus* Boiss. ex DC., and *Sideritis carbonellii* Socorro.

Ex-situ conservation measures are identical for most plant species. For *Crepis granatensis*, there are seeds in the Andalusian plant germplasm bank. It would be advisable to collect and store more seeds in the germplasm bank to largely preserve the genetic diversity and evolutionary potential of the species.

Reinforcement, reintroduction and translocation programs have to be carefully studied and applied. According to IUCN (2013), those species with a high risk of extinction have priority in terms of assessing and performing such programs. *Crepis granatensis* is an ideal narrow endemic species for implementing this type of measures. However, before conducting any action, many factors such as threats that will face the species, like ecological, economic and social aspects of the selected territory, and origin and genetic diversity of the plant material (Gordon 1994) must be taken into account. For instance, in the case of *C. granatensis*, the reinforcements of natural populations should be carefully chosen (taking into account the aforementioned factors), as well as the optimal areas for reintroductions and translocations. Thus, Sierra Cazorla and Sierra

Segura can be suitable enclaves for reintroduction as some authors consider this species to be extinct in recent times (Blanca et al. 1987, 2003).

All these aforementioned measures should be considered both in the current recovery plan and management actions for the species. Further studies are necessary to conduct using other molecular techniques as well as other approaches within conservation biology such as demography, pollination and reproductive biology, modelling, and phylogeny and phylogeography. Likewise, this study is part of an ongoing multidisciplinary project that not only takes into account *C. granatensis* but also other threatened screes species for which there is not much information at present.

## Conclusions

The results of the AFLP analysis on the current *C. granatensis* populations show a pattern of high within-population diversity but low among-population and among region divergences. Two clusters were identified: one corresponds to SAGRA population, and the other corresponds to Mágina populations. We found low genetic differentiation and moderate-high gene flow between populations in the same region and, vice versa, between regions. These patterns of genetic diversity and levels of genetic differentiation may be accounted for by entomophilous outcrossing between nearby populations, despite the fragmented distribution of them, avoiding genetic impoverishment. Long-distance pollen dispersal events may sporadically occur between regions. On the contrary, long-distance seed dispersal events seem unlikely to occur. All populations contain high values of genetic diversity, which involve protecting them equally as important genetic pools. This study suggests that *C. granatensis* is not threatened due to genetic factors but ecological and biological factors (especially its high specificity to the habitat, decreasing its ecological plasticity). *In-situ* and *ex-situ* conservation measures

should be implemented to preserve the evolutionary potential of the species. Finally, new insights provided in this study should be considered for updating the recovery and conservation plan and implementing management actions for the species.

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Table 1. Geographical location, number of sampled individuals (n), percentage of polymorphic loci (PLP), expected heterozygosity (Hj), average gene diversity within populations (Hw), total genetic diversity (Ht), frequency down-weighted marker values (DW), genetic differentiation among populations (Fst) of the populations of *C. granatensis*.

| Population code | Geographical coordinates | n | PLP | Hj | Hw | Ht | DW | Fst |
|---|---|---|---|---|---|---|---|---|
| SAGRA | 37º 57' 19.6" N, 2º 33' 29.9" W | 31 | 77.4 | 0.258 | | | 714.8 | |
| MAG1 | 37º 43' 29.5" N, 3º 28' 48.3" W | 18 | 63.9 | 0.221 | | | 127.3 | |
| MAG2 | 37º 44' 10.6" N, 3º 30' 51.6" W | 18 | 58.4 | 0.202 | | | 85.1 | |
| MAG3 | 37º 44' 48.3" N, 3º 28' 32.7" W | 33 | 64.8 | 0.217 | | | 258.1 | |
| Total | | | | | 0.225 | 0.260 | | 0.137 |

Table 2. Analysis of molecular variance (AMOVA) among and within populations and among regions of *C. granatensis* based on AFLP data. df: degree of freedom, SS: sum of squares, Est. Var.: estimated variance, % variation: percentage contribution of each component in relation to total variation, P-value* of fixation index after 10000 random permutations.

| Source of variation | df | SS | Est. Var. | % variation | P-value* |
|---|---|---|---|---|---|
| Among Regions | 1 | 2.527 | 0.042 | 18% | <0.001 |
| Among Populations | 2 | 1.167 | 0.019 | 8% | <0.001 |
| Within Populations | 96 | 16.212 | 0.169 | 74% | <0.001 |
| Total | 99 | 19.906 | 0.230 | 100% | |

Table 3. Fst pairwise between populations of *C. granatensis*

| | SAGRA | MAG1 | MAG2 | MAG3 |
|---|---|---|---|---|
| SAGRA | 0.000 | 0.205 | 0.151 | 0.193 |
| MAG1 | 0.205 | 0.000 | 0.0923 | 0.041 |
| MAG2 | 0.151 | 0.093 | 0.000 | 0.085 |
| MAG3 | 0.193 | 0.041 | 0.085 | 0.000 |

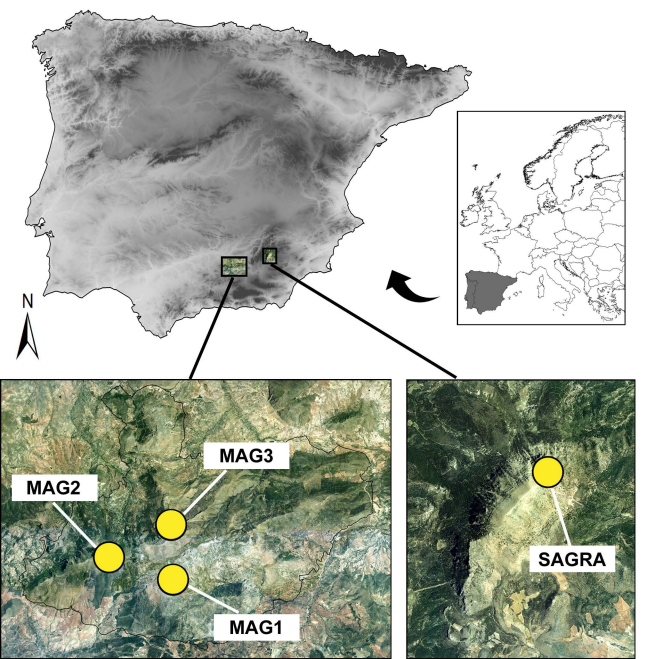
Table 4. Gene flow (Nm) pairwise between populations of *C. granatensis*

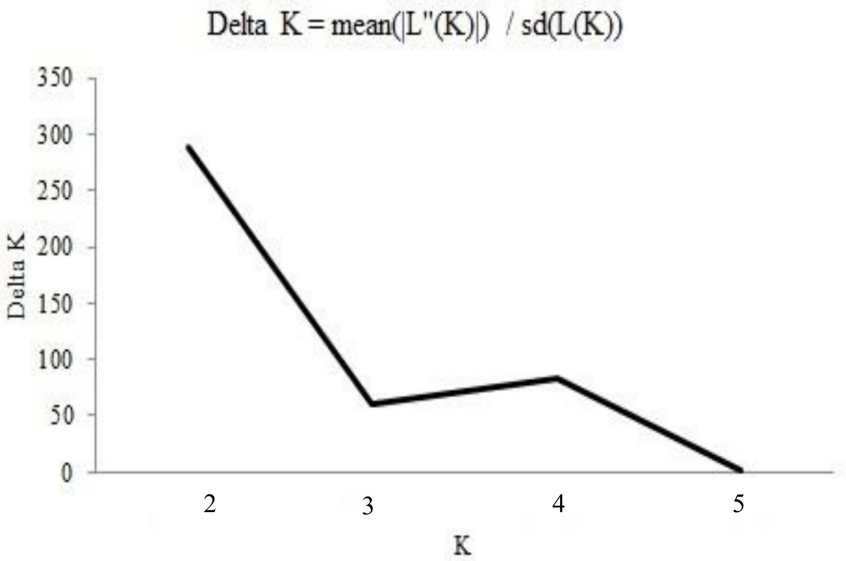
| | SAGRA | MAG1 | MAG2 | MAG3 |
|---|---|---|---|---|
| SAGRA | 0.000 | | | |
| MAG1 | 0.967 | 0.000 | | |
| MAG2 | 1.403 | 2.450 | 0.000 | |
| MAG3 | 1.047 | 5.908 | 2.688 | 0.000 |

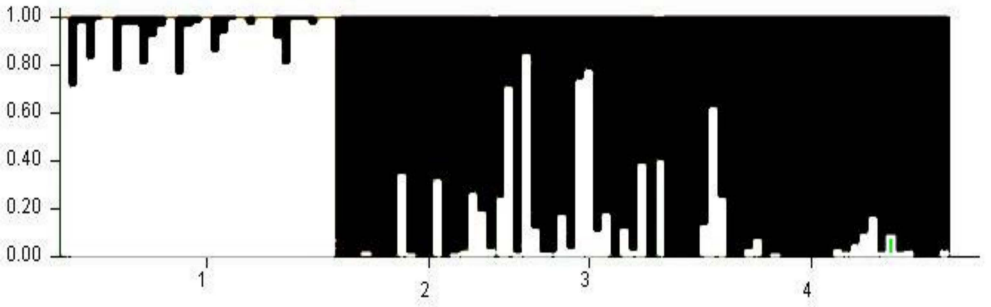
Figure 1. Geographic location of the study populations representing the whole distribution range of *Crepis granatensis*. Habitat and flowering plant are also displayed.

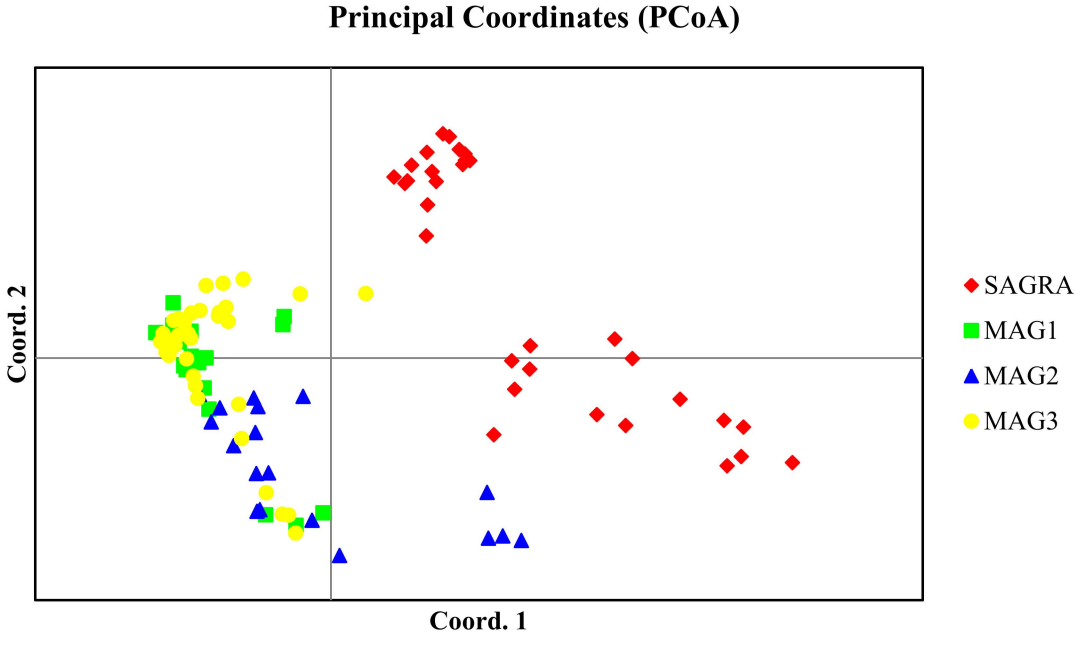
Figure 2. Bayesian analysis of the population genetic structure using the software STRUCTURE assuming K = 2. Each bar represents a single individual, with colours indicating different genetic contribution of each detected cluster in the mixture analysis.

Figure 3. Principal Coordinates Analysis (PCoA) of the 100 individuals from four study populations of *C. granatensis* based on pairwise Nei's (1978) genetic distances.


N
MAG3
MAG2
MAG1
SAGRA


Delta K = mean(|L''(K)|) / sd(L(K))
350
300
250
200
150
100
50
0
Delta K
2
3
4
5
K


1.00
0.80
0.60
0.40
0.20
0.00
1
2
3
4


Principal Coordinates (PCoA)
Coord. 2
Coord. 1
SAGRA
MAG1
MAG2
MAG3