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Spatial genetic structure and genetic diversity of a natural population of *Ramorinoa girolae* in San Juan province (Argentina): An exploratory analysis

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Abstract. *Ramorinoa girolae* Speg. (chica) is an endemic tree species which belongs to the Argentinian xerophyte flora. This species constitutes an appreciated forest resource for local people due to its fruits (a substantial basis of the diet of local communities) and its timber (one of the hardest woods). Moreover, chica was pointed as a vulnerable species because it is a monotypic species with low abundance and a very restricted endemism (San Juan, San Luis and La Rioja provinces). There is limited information concerning the status of genetic diversity of the species. For this, we performed an exploratory analysis to characterize the genetic diversity of a natural population located near Ischigualasto Provincial Park (San Juan, Argentina) using AFLP molecular markers in 19 individuals. The genetic data were correlated with morphometric, ecological and geographical data. Our results suggest that this population exhibited a high genetic diversity (Pj=82.3%). These values also were correlated with geographical matrices, revealing that this population exhibited a particular spatial genetic structure (SGS; Mantel Test r=0.45, $P<0.001$) and spatial autocorrelation. In addition, values of genetic similarity among the different analyzed individuals revealed the absence of clones, suggesting a significant contribution of genetic variability due to sexual reproduction in the studied population.

[Keywords: tree endemic specie, desert Monte, genetic polymorphism, AFLP molecular markers]

Resumen. Estructura genética espacial y diversidad genética de una población natural de *Ramorinoa girolae* en la provincia de San Juan (Argentina): un análisis exploratorio. *Ramorinoa girolae* Speg. (chica) es un árbol endémico nativo de la flora xerófita argentina. A esta especie se la considera un recurso forestal muy apreciado debido a sus frutos (que constituyen una parte importante de la dieta de los pobladores locales) y su madera (una de las más duras). Además, la chica fue clasificada como una especie vulnerable porque es una especie monotípica de abundancia baja, con un endemismo muy restricto (limitado a las provincias de San Juan, San Luis y La Rioja) y con poca resistencia al fuego. Dado que existe escasa información sobre el estatus de la diversidad genética de la especie, realizamos un análisis exploratorio para caracterizar la diversidad genética de una población natural ubicada en la proximidad del Parque Provincial Ischigualasto (San Juan, Argentina) por medio de marcadores moleculares AFLP en 19 individuos. Los datos genéticos se correlacionaron con datos morfométricos, ecológicos y geográficos. Nuestros resultados sugieren que esta población posee una diversidad genética elevada (Pj=82.3%). Además, estos valores se correlacionaron con matrices geográficas, lo que reveló que la población exhibió una particular estructura genética espacial (SGS, Prueba de Mantel r=0.45; $P<0.001$). Por otra parte, los valores de similitud genética entre los diferentes individuos analizados revelaron la ausencia de clones, lo que sugiere una contribución significativa de la variabilidad genética producto de la reproducción sexual en la población estudiada.

[Palabras clave: especie arbórea endémica, desierto del Monte, polimorfismo genético, marcadores moleculares AFLP]

Introduction

Several factors, such as climate change and habitat modification, are the immediate causes of species becoming endangered (Murray 2002). In the last past centuries, these factors led to the extinction or geographic ranges confinement of many plant species (Wiens 2016). Rare and endemic plant taxa have many attributes that render them more vulnerable to extinction than others (Van Dyke 2003; Farnsworth et al. 2006). Human activity can modify the entire extent of their narrow geographic range, leading to their extinction. Under this scenario, understanding the population status, genetic diversity, and genetic structure of threatened species are the first steps in determining management and conservation policies in the short and long term.

To develop appropriate recovery and conservation management plans, an understanding of species biology and ecology

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as well as knowledge of the environmental forces responsible for shaping genetic diversity are crucial (Neel and Ellstrand 2003). Genetic diversity is an important consideration in species conservation because it influences a population's ability to adopt to a changing environment (Jump et al. 2009; Kirk and Freeland 2011). Genetic drift and inbreeding are the two mechanisms operating in small populations that influence their genetic variation, whereby genetic variation is determine by the joint action of natural selection and genetic drift. Whereas inbreeding reduces reproduction and survival, the loss of genetic variation decreases the ability of populations to cope with environmental change (Frankham et al. 2002). Decreased heterozygosis characterizes populations of threatened species, which are prone to suffer from local extinction caused by genetic factors (Spielman et al. 2004). In small populations, the relative importance of genetic drift is higher; deleterious alleles can become more frequent and fixed in a population by chance, resulting in the loss of genetic variability.

To date, genetic studies in arboreal species have only been performed for a limited number of species (Lacerda et al. 2001; Juárez-Muñoz et al. 2002; Casiva et al. 2002; Juchum et al. 2007; Kumar et al. 2015; Martínez Araneda et al. 2011). One of the main causes of the scarce of studies is the absence of molecular tools and genetic markers applicable to a wide range of species. Currently, no universal marker system is available for codominant nuclear loci. Such markers need to develop separately for each species, and can be extremely costly in resources and time (Squirrel et al. 2003). These technical limitations lead to the broad application of random amplification techniques for assessing genetic diversity such as AFLPs (amplified fragment length polymorphism) (Vos et al. 1995). Despite the disadvantage that this molecular marker is dominant, it allows an assessment of genome-wide variation consisting largely of non-coding DNA (Wong et al. 2001).

The Monte Ecoregion includes the strip of more arid lands of Argentina, which constitute fragile environments. It is characterized by strong environmental limitations such as extreme temperatures, seasonal and daily high temperature amplitude, scarce rainfall, high irradiation and low primary productivity, among others (Morello 1958). Despite these limitations, arid environments harbor a rich biodiversity of flora and fauna with adaptations to the extreme conditions, and a high number of endemic species with conservation problems (Mares 1992; Roig-Juñent et al. 2001; Poll et al. 2005). Among them, woody species (trees and shrubs) play an important role in the regulation of ecosystem processes as they contribute to the functioning and maintenance of the physical integrity of ecosystems (Gutiérrez and Squeo 2004). However, the action of natural and anthropic agents has leaded most area of the Monte to a moderate to severe status of desertification (Poll et al. 2005; Villagra et al. 2009), threatening its biodiversity.

One of the most vulnerable woody species is *Ramorinoa girolae*, locally called chica, a focus of interest in this study. It is an exclusive aphyllus legume of the xerophilous flora of Monte of mountains and basins eco-region (Brown et al. 2006), with an endemism of distribution very restricted to the provinces of San Juan, San Luis and La Rioja (Hadad et al. 2014; Zapata 2017). It is also the only representative species of the genus (monotypic) with a high degree of taxonomic singularity (Kiesling 1994; Zapata et al. 2017), and was classified as threatened (Category 4 on a scale of 1 to 5) (PlanEAr 2008). To date, basic aspects of its reproductive biology are unknown, limiting the application of management and conservation strategies. *R. girolae* allocates a lot of resources in fruit and seed production although a high percentage of them suffers predation during the pre-dispersal immature stage (58% are depredated by the larvae of *Anypsipyla univitella*) (Papú et al. 2015) and mature stage (*Octomys mimax*) (Campos and Giannoni 2013). On the other hand, due to the fact that it is rare to find seedlings or young plants (Hadad et al. 2014), one widespread accepted strategy of multiplication in this species is the asexual propagation (Femenía and Giménez de Bolzón 1992). It is common to find long-lived and outsized trees of *R. girolae* with a crown tree extension higher than 30 m (named as chica patch) accompanied by smaller nearby specimens that are apparently independent from them.

One of the main questions to be answered in this study is to determine the degree of genetic similarity among pairs of closely spaced plants in order to establish if they are clones or genetically different individuals. We hypothesize that most of the closely spaced

individuals are genetically similar, resulting from clonal reproduction. Additionally, we expected to find low genetic polymorphism as observed in most rare and low-density species.

MATERIALS AND METHODS

Data collection

This study was carried out on natural populations of *R. girolae* (Figure 1) located in the site of "Mina de Cuarzo" (IPP, 30°12´12´´ S - 68°00´01´´ W) in San Juan province. This site was selected because it has high density of chica individuals of different sizes, the proximity to a protected Ischigualasto Provincial Park and the multiple sources of potential disturbance such as contiguity to a quartz mine, forest resource exploitation and the recent construction of National Road 150, which crosses the park. Sample collection was realized on March (2013), following the natural distribution of the species along two confluent dry riverbeds.

R. girolae is associated with rocky hillsides (Hadad et al. 2014), and its distribution is restricted to some aspects in the slopes (Márquez et al. 2005). The species constitutes one of the few arboreal species in the Monte of mountains and basins eco-region belonging to the chica community (Acebes et al. 2010).

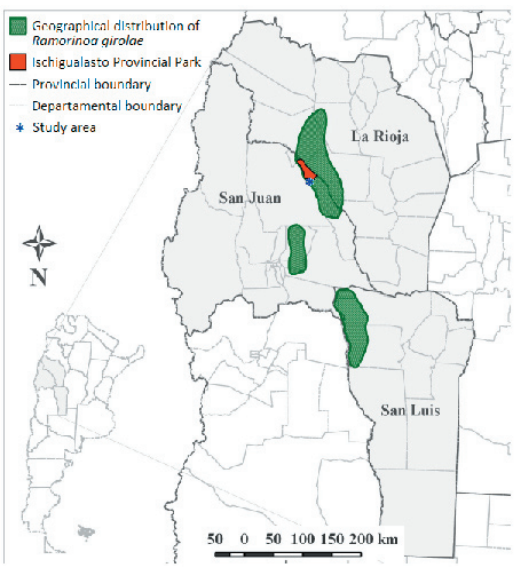

Figure 1. Geographical distribution of *Ramorinoa girolae* and location of Ischigualasto Province Park (San Juan, Argentina). Sources: COFEMA-SIFAP, APN, SAyDS.

Figura 1. Distribución geográfica de *Ramorinoa girolae* y localización del Parque Provincial Ischigualasto (San Juan, Argentina). Fuentes: COFEMA-SIFAP, APN, SAyDS.

Chical is one of most rich in plant species, is the most heterogeneous community of study site characterized by high cover of rocky and steep terrain (Acebes et al. 2010). As an arboreal species, it plays an important role in regulation of ecosystem processes and maintaining their physical integrity. Thereby, it was reported that chica reduces soil erosion, produces hydraulic lift from deeper groundwater, retains moisture and redistributes soil nutrients (Femenía and López 2004). In association with this species plant, there are epiphytes, succulents and a large number of species of mammals, birds and insects (Campos et al. 2017) (Figure 2).

Individuals separated from each other by at least 50 m were selected according to accessibility along a dry riverbed because of the irregular topography of site. Each

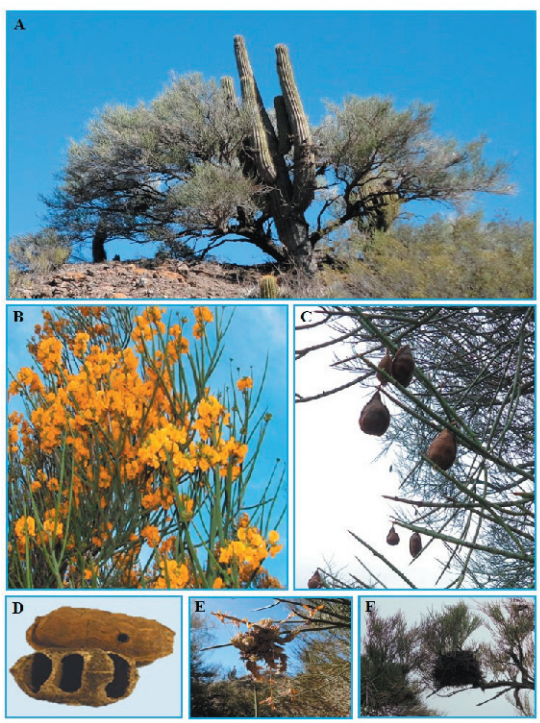

Figure 2. *Ramorinoa girolae* and its association with other species. a) The association with *Trichocereus terscheckii* is very common, behaving *R. girolae* as a nurse plant of plantlets of this specie. b) Flowers are visited by different species of insects. c) *R. girolae*'s fruits consumed by *Anypsipyla univitella* and d) *O. mimax*. e) Epiphyte *Tillandsia angulosa.* f) Bird's nest set on the branches of *R. girolae*.

Figura 2. *Ramorinoa girolae* y su asociación con otras especies. a) La asociación con *Trichocereus terscheckii* es muy común, y *R. girolae* se comporta como planta nodriza de plántulas de esta especie. b) Las flores son visitadas por diferentes especies de insectos; c) los frutos de *R. girolae* son consumidos por *Anypsipyla univitella* y d) *Octomys mimax*. e) Epifita *Tillandsia angulosa*. f) Nido de pájaro en las ramas de *R. girolae*.

individual was considered as a sampling unit, and its geographical localization was recorded using Universal Transversal de Mercator (UTM) and altitude (m) with a GPS. The morphological quantitative traits measured were: H, tree tallness (m); diameter of the tree crown (D); and eqBD, equivalent Basal Diameter (cm). The eqBD was calculated according to Álvarez et al. (2006) with:

$$eqDB = 2 \sqrt{\{\pi (dab_1/2)2+\pi (dab_2/2)2+\pi (dab_3/2)2+....+\pi (dab_n/2)2\}/\pi}$$

where: dab_1, dab_2, …, dab_n are the basal diameters of each branch.

Tissue samples of young thin herbaceous shoots of the year from the selected individuals were collected and maintained at 4 °C until laboratory DNA extraction.

In *R. girolae*, despite countless expert attempts, dating using the method of counting the tree rings has proved to be unsuccessful(Martin Hadad, CIGEOBIO, personal communication). In order to establish relationship among plants, the selected individuals were classified according their size with the following criterion: 1) renewals (eqDB<1 cm), 2) juveniles (eqDB<5 cm), 3) small-sized adults (5 cm ≤ eqDB<20 cm), 4) medium-size adults (20 cm ≤ eqDB< 70 cm) and 5) oversized adults or chica patch (eqDB ≥ 70 cm). Thus, we assume that the age of the individual plants will be increasing along this scale.

The criterion of selection of groups of plants was the presence of an individualized small-size plant (e.g., renewals, juveniles or small-sized adults) distanced at less than 20 m from patches or an oversized plant. In the cases where small plant size was absent, two oversized plants distanced at less than 20 m were sampled. Isolated plants were also collected downhill from the dry riverbed. To estimate genetic identity within a patch, we collected tissue samples from opposite branches. In total, 19 individuals were sampled in this exploratory analysis.

Genetic analysis

In the laboratory, steams were cut with a scalpel into thin slices and ground in a thin powder with liquid nitrogen. DNA extraction was performed using the CTAB protocol (Doyle and Doyle 1987). We employed the technique of Amplified Fragment Length Polymorphism (AFLP) as molecular marker to perform genetic analysis. It was selected for several reasons such as: 1) its robustness and repeatability, 2) a high detection power of genetic variability and polymorphism within few reactions and 3) generation of genetic information in species as *R. girolae* in which genomic information is not available. The protocol was performed following Vos et al. (1995) with minor modifications. For the restriction reactions, 300 ng of DNA, 2 µL of buffer M, 2 units of EcoRI (Roche), 0.5 units of Tru9I (Roche) in a 20 µL of final volume were restricted at 37 °C for 3 h and enzymes were then inactivated at 65 °C for 20 minutes. The following steps were performed independently on each digestion (n=2).

Adapters ligation was performed with 10 µL restriction reaction, 1.25 µL of EcoRI adapter (5 pmol/µL), 1.25 µL of MseI adapter (50 pmol/µL) (Table 1), 2 µL of T4 ligase buffer 10×, 0.4 units of T4 ligase (Roche) in a final volume of 20 µL, for 10 h at 20 °C and enzymes were then inactivated at 70 °C for 10 minutes. The ligation products were diluted 10-fold. Pre-amplification was conducted with 2.5 µL from the previous dilution, 2 µL PCR buffer 10×, 0.6 µL $MgCl_2$ (50 mM), 0.2 µL primer EcoRI +1 (50 ng/µL), 0.2 µL primer MseI +1 (50 ng/µL) (Table 1), 0.4 µL of dNTPs (5 mM) and 1 unit of Taq polymerase (Bionac TaqUBA), in a final volume of 20 µL. Reactions were carried out for 20 cycles of 30 s at 94 °C, 60 s at 56 °C and 60 s at 72 °C. Pre-amplification products were diluted threefold. For the selective amplification, fifteen different combination of primers were selected (Table 1). Amplification was conducted with 1 µL of the diluted preamplification, 0.5 µL EcoRI +3 primer (50 ng/µL), 0.6 µL MseI +3 primer (50 ng/µL) (Table 1), 0.8 µL dNTPs (5 mM), 2 µL PCR buffer (10×), 0.6 µL MgCl2 (50 mM), 1 unit of Taq polymerase (Bionac TaqUBA),

Table 1. Oligonucleotides used for AFLP analysis.

Tabla 1. Oligonucleótidos utilizados para el análisis de AFLP.

Type of primer	Sequence
EcoRI adapter	5′-CTCGTAGACTGCGTACC
	CTGACGCATGGTTAA-5′
EcoRI +1 primer	5′-AGACTGCGTACCAATTC**A**
EcoRI +3 primers	E + ACG
	E + ACA
MseI adapter	5′-GACGATGAGTCCTGAG
	TACTCAGGACTCCAT-5′
MseI + 1 primer	5′-GACGATGAGTCCTGAGTAA**C**
MseI +3 primers	M + CTG
	M + CTA
	M + CAA
	M + CAT

in a final volume of 20 µL. The touchdown program consisted in 1 cycle of 30 s at 94 °C, 30 s at 65 °C and 60 s at 72 °C, decreasing the annealing temperature by 0.7 °C per cycle during 12 cycles, and 23 cycles for 30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C. In all the steps, the quality and integrity of the DNA and digestion reactions was monitored by electrophoresis in 1% agarose gel (w/v) at constant 90 volts during 40 minutes.

The amplified fragments of AFLP were resolved in 6% polyacrylamide DNA sequencing gel and staining with silver nitrate according to Bassam and Caetano-Anollés (1993). Fragments from AFLP polyacrylamide gels were scored into a binary character matrix indicating presence (1) or absence (0). Only fragments within the 200-600 bp range were scored. From the binary genetic matrix, we determined the following genetic diversity indexes: Polymorphism (Moreno Vázquez 2001), Uniformity (Weising et al. 1995) and Nei´s Genetic Diversity (Nei 1973).

Genetic distances and spatial structure

The genetic distances among individuals was calculated from the Dice´s Genetic Similarity (G_S) (Dice 1945) using the formula sqrt (1-G_S). Genetic associations between individuals were determined by cluster analysis using the Ward's minimum variance method. The spatial genetic structure (SGS) was determined by correlating the genetic and the geographical coordinates distance matrices using the Mantel Test (Mantel 1967). Additional correlations with morphological parameters were also performed. In all cases, the correlation matrix was estimated using standardized data and calculating the eigenvalues and projected data graphically. All these analyses were performed using the program InfoStat version 2017 (Di Rienzo et al. 2017).

For the spatial autocorrelation analysis, which measures correlations between pairs of individuals as a function of distance, the autocorrelation coefficient r was determined and results were summarized by a correlogram. We used the Spatial package in GenAlEx 6.501 (Peakall and Smouse 2012) with the Single Populations and Variable Distance Class options. The 95% confidence interval about the null hypothesis of non-spatial structure for the combined data set was determined by 999 permutations of the data. The 95% confidence interval about each r-value was determined by 999 bootstrap resamplings. As recommended by Banks and Peakall (2012), we considered a result as significant when $P<0.01$.

RESULTS

Morphological characterization of individuals

Despite the attempt to collect sapling and/or juvenile individuals, they were absent in the sampled site. Six groups of plants composed of adult individuals of different sizes were sampled along two confluent dry riverbeds. Fifteen percent of relieved specimens were adults of small sized, the individuals 11, 14 and 16 (eqDB=19.0±14.0 cm; H=1.3±0.8 m; D=0.8±0.2 m). This category was followed by adult medium-size trees (eqDB=56.0±10.0 cm; H=3.1±0.6 m; D=9.6±4.4 m), that constitutes 21% of sampled trees (individuals 1, 2, 5 and 17). The remainder individuals observed and sampled in this research (65%) corresponded to decumbent oversized patches (eqDB=93.6±5.2 cm; H=5.7±0.3 m; D=18.7±2.3 m).

Genetic analysis

In total, we obtained 164 AFLP molecular markers, from which 82.3% resulted polymorphic among individuals (Table 2). From this data, the estimation of Nei´s Genetic Diversity Index (H_e) was 0.28±0.04 whereas the Uniformity Index (U_j) was 0.49. The estimation of G_S among pairs of individuals according to Dice Coefficient revealed that 92% of the individuals share 0.75-0.90 G_S (Figure 3). The lowest values of G_S ranged between 74-75% (pair of individuals: 11-13, 11-5, 11-14, 11-18 and 18-12) (Table 3). Towards the other end, the highest value of G_S was 99%, and comes from samples collected from branches belonging to the same patch.

High values of G_S ranged between 90-91% were found among different sized nearby plants (11-08 and 13-14, distanced at 15 and 9 m, respectively). However, pairs of individuals along the runway with high G_S, such as 15-10

Table 2. Number and percentage of polymorphic fragments detected in AFLP binary matrices of *R. girolae*.

Tabla 2. Número y porcentaje de fragmentos polimórficos detectados en la matriz binaria de AFLP de *R. girolae.*

Type of AFLP fragment	Total	Polymorphism (%)
Monomorphic	29	17.7
Polymorphic	135	82.3
Total	164	100.0

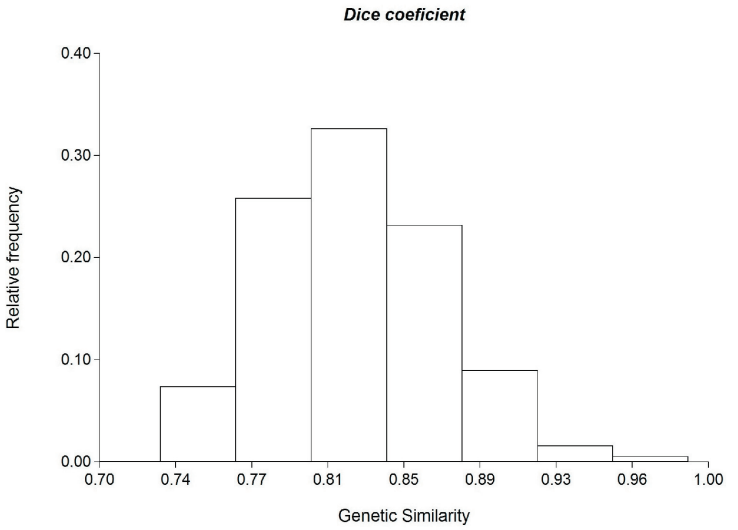

Figure 3. Frequency distribution of 164 AFLP genetic similarity coefficients among 19 individuals of *R. girolae*.

Figura 3. Distribución de las frecuencias de los coeficientes de similitud entre los 19 genotipos de *R. girolae* obtenidos a partir de los 164 marcadores AFLP.

Table 3. Genetic similarity coefficient (G_S) among 19 individuals of *R. girolae* obtained by AFLP markers (Dice coefficient).

Tabla 3. Coeficientes de similitud genética (G_S) entre 19 individuos de *R. girolae* obtenidos por marcadores AFLP (coeficiente de Dice).

RG	1	10a	10b	11	12	13	14	15	16	17	18	19	2	20	4	5	6	7	8	9
1	1																			
10a	0.87	1																		
10b	0.85	0.99	1																	
11	0.77	0.77	0.75	1																
12	0.84	0.87	0.86	0.80	1															
13	0.82	0.86	0.85	0.73	0.89	1														
14	0.84	0.91	0.92	0.74	0.86	0.91	1													
15	0.89	0.91	0.90	0.82	0.86	0.87	0.89	1												
16	0.76	0.79	0.78	0.78	0.79	0.79	0.79	0.87	1											
17	0.76	0.78	0.76	0.78	0.82	0.79	0.78	0.85	0.85	1										
18	0.76	0.76	0.75	0.74	0.81	0.81	0.82	0.86	0.86	0.86	1									
19	0.83	0.85	0.85	0.77	0.81	0.83	0.84	0.91	0.85	0.82	0.86	1								
2	0.88	0.80	0.80	0.78	0.83	0.79	0.81	0.85	0.81	0.75	0.74	0.76	1							
20	0.84	0.85	0.85	0.77	0.86	0.87	0.88	0.92	0.84	0.83	0.85	0.93	0.79	1						
4	0.82	0.81	0.80	0.79	0.84	0.83	0.83	0.87	0.81	0.77	0.79	0.78	0.88	0.82	1					
5	0.84	0.83	0.82	0.74	0.86	0.86	0.85	0.84	0.79	0.77	0.78	0.79	0.84	0.83	0.87	1				
6	0.80	0.82	0.80	0.80	0.82	0.81	0.81	0.87	0.78	0.80	0.80	0.82	0.78	0.83	0.81	0.88	1			
7	0.78	0.78	0.78	0.79	0.82	0.84	0.82	0.87	0.81	0.82	0.85	0.79	0.80	0.82	0.84	0.82	0.86	1		
8	0.81	0.83	0.81	0.90	0.87	0.85	0.83	0.84	0.79	0.83	0.84	0.80	0.83	0.81	0.80	0.81	0.85	0.85	1	
9	0.85	0.89	0.89	0.80	0.87	0.86	0.90	0.88	0.80	0.80	0.80	0.84	0.83	0.83	0.87	0.84	0.85	0.82	0.87	1

(G_S=0.91; 300 m); 14-10 (G_S=0.91; 136 m); 9-14 (G_S=0.9; 197 m) and 19-20 (G_S=0.93; 50 m) were also found.

Spatial genetic structure

Cluster analysis of genetic polymorphism revealed 4 different genetically related groups of individuals spatially arranged along the riverbed, suggesting that the nearest individuals are more related genetically than those distanced geographically (Figure 4). Moreover, the analysis of SGS by Mantel test showed a significant positive correlation (r=0.45, P<0.001) between the genetic and geographical distances (Figure 5). The correlation analysis of the genetic against the morphological matrixes also revealed a significant positive relationship (r=0.22, P<0.035).

Spatial autocorrelations analysis showed highly significant deviations from the null hypothesis of no correlation between genetic similarity and pairwise distance (Figure 6). This revealed that r-values are higher among individuals that are closer to each other (distance class 50 m) and decrease at a greater distance, being non-significant at distances higher than 150 m.

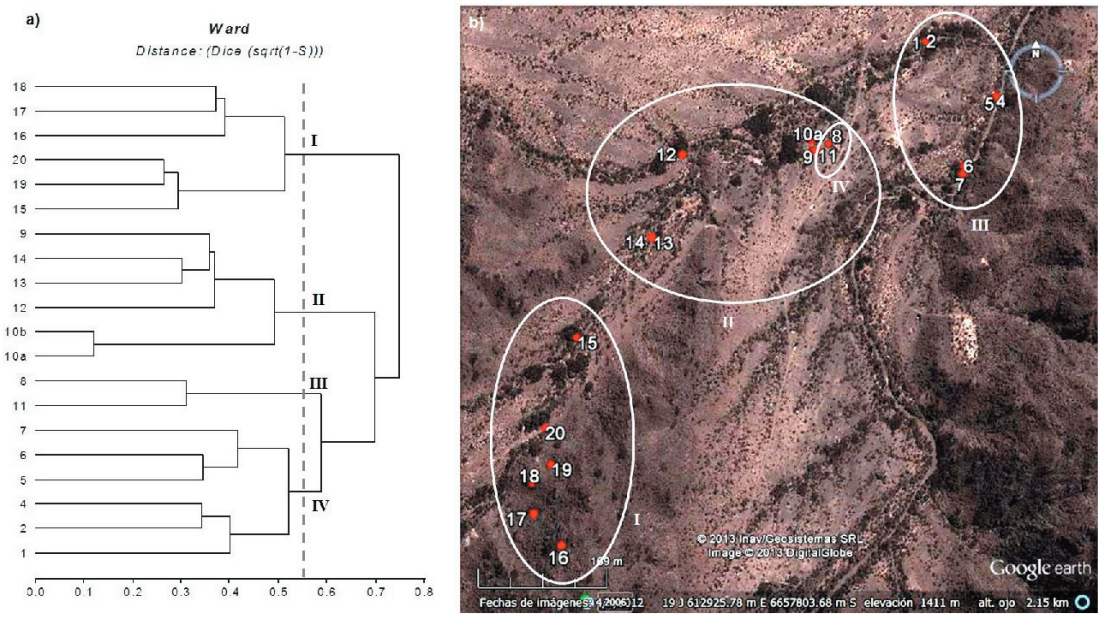

Figure 4. Genetic and spatial distance among 19 individuals of *R. girolae*. a) Dendrogram obtained by cluster analysis in base of 164 AFLP fragments using Dice´s matrix of similarity and Ward method. Four distinct groupings (I, II, III and IV) were evident with a Dice coefficient of 0.55 (dotted line). b) Satellite image of the study area showing the two confluent drier riverbed and the position of individuals of *R. girolae* (indicated with points). Circles indicates groups of individuals related genetically.

Figura 4. Distancia genética y espacial entre los 19 genotipos de *R. girolae*. a) Dendrograma obtenido por análisis de agrupamiento en base a 164 fragmentos de AFLP utilizando la matriz de similaridad de Dice y el método de Ward. Se evidenciaron cuatro grupos distintos (I, II, III y IV) con un coeficiente de Dice de 0.55 (línea punteada). b) Imagen satelital del área de estudio mostrando los dos ríos secos confluyentes y la posición de los genotipos de *R. girolae* (indicados con puntos). Los círculos indican los grupos de genotipos relacionados genéticamente.

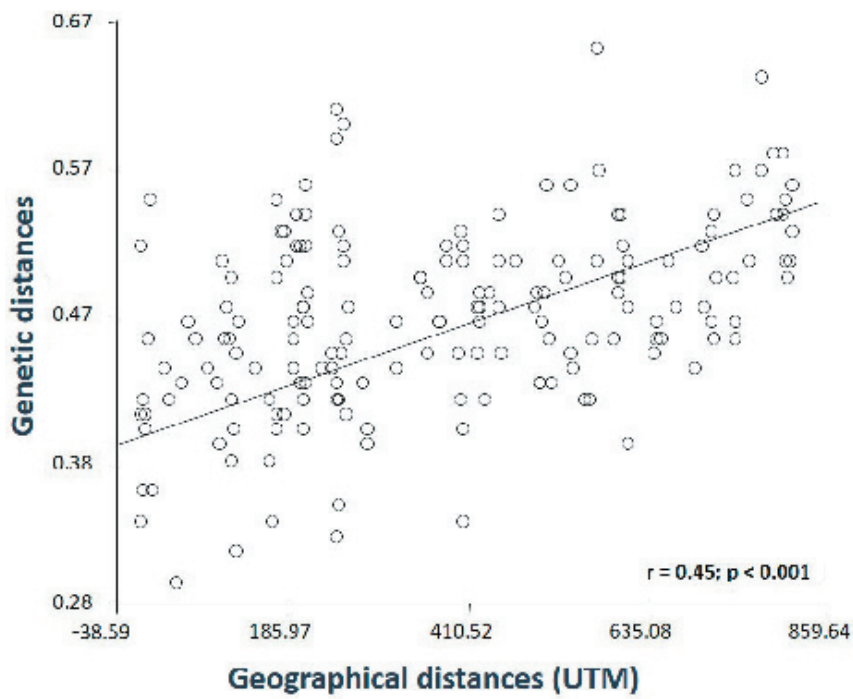

Figure 5. Spatial genetic structure of individuals of *R. girolae*. Scatter plots of the pairwise genetic distance (Dice, sqrt(1-G_S)) versus the geographical distance (UTM) of all sampled individuals of *R. girolae*. A significant positive relationship (r=0.45, *P*<0.001) by Mantel test was observed.

Figura 5. Estructura genética espacial de genotipos de *R. girolae*. Gráficos de dispersión de los pares de distancias genética (Dice, sqrt (1-GS)) versus la distancia geográfica (UTM) de todos los genotipos de *R. girolae*. Se observó una relación positiva significativa (r=0.45; *P*<0.001) por la prueba de Mantel.

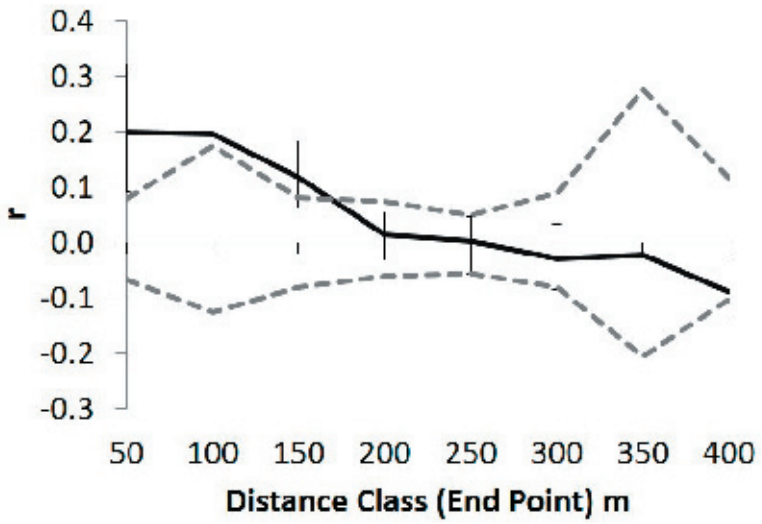

Figure 6. Genetic spatial autocorrelation of *R. girolae* individuals and geographical distance (m). Solid line=r, the autocorrelation coefficient among individuals in a given distance class in meters. Dashed lines indicate the upper and lower 95% confidence interval around the null hypothesis of "no spatial structure" for the data set as determined by 999 permutations of the data. Error bars indicate the 95% confidence interval about each r value, determined by 1000 bootstrap re-samplings.

Figura 6. Autocorrelación genética espacial de individuos de *R. girolae* y distancia geográfica (m). Línea sólida=r, el coeficiente de autocorrelación entre genotipos representado en clases de distancia en metros. Las líneas discontinuas indican los límites superior e inferior de los intervalos de confianza del 95% alrededor de la hipótesis nula de "inexistencia de estructura espacial" para el conjunto de datos determinado por 999 permutaciones de los mismos. Las barras de error indican el intervalo de confianza del 95% para cada valor de r, determinado por 1000 muestreos por bootstrap.

Discussion

Some reproductive aspects of R. girolae

Many woody plants combine reproduction by seed with some level of asexual spread, and the latter may have important consequences on spatial genetic distribution, particularly at a local level. Restricted gene dispersal arises not only from seed and from pollen, but also from spatial displacement of vegetative units (Gliddon et al. 1987) that is a function of the mode of vegetative spread. In long-lived woody plants, clonal spread is common by re-sprouting and the degree of clonal spread will depend on the origin of re-sprouts (basal burls, crowns and lignotubers, or roots) and on the number of generations of asexual spread (Dodd et al. 2013). In the case of *R. girolae*, sexual reproduction occurs by seed dispersions. Asexual reproduction is supposed to occur when the branches are buried by pebble transport by alluvial rivers during the spring, thus originating independent roots, branches and, eventually, new plants (Femenía and López 2004). Therefore, plants close to each other could proceed from either of these two propagation mechanisms. Our results suggest that, contrary to expectations, small size plants grown in the closeness of chica patches or oversized specimens do not constitute ramets, but they are genetically different individuals probably resulting from sexual reproduction. In addition, despite the fact that we have found levels of considerably high genetic similarity among the sampled plants (G_S range: 0.73-0.91) (Figure 3), no clones were identified.

There are two main factors that can induce the high levels of G_S observed in *R. girolae*: 1) overestimation by homoplasy due the use of dominant markers and 2) the occurrence of self-pollination. Concerning the first option, the frequency of erroneous scoring of non-homologous bands of similar mobility in dominant markers may increase for genetically less similar individuals, particularly with increasing taxonomic ranks (Kremer et al. 2005; Meudt and Clarke 2007). However, in the AFLP technique this phenomenon is less likely to occur because of high gel resolution. Moreover, sampled individuals of *R. girolae* belong to the same population, reducing the probability of homoplasy. Bearing this in mind, the second option of self-pollination

is the most plausible reason for the observed high G_S. Values of G_S among self-pollinated species are generally high and range among 0.737-0.980 in rice (Aggarwal et al. 2002), 0.66-1.00 in *Solanum kurtiznum* (Hidalgo 2013) and 0.632-1.000 in *Phaseolus vulgaris* (Beharav et al. 2010). In the case of *R. girolae*, despite there is scarce information concerning basic aspects of its reproductive mechanisms, some prelaminar pieces of evidences suggest that this species has cross-fertilization and requires pollinators for fruit set (Zapata 2017). Thus, our results might suggest that the species is self-compatible in a similar manner of most of Fabaceae (Rodriguez-Riaño et al. 1999). However, deeper analysis is required to confirm this issue.

The high G_S identified among distant genotypes reinforces this observation of absence of clonal propagation. Several factors have been associated to fruit and seed dispersion in the space, such as gravity, water runoff, and activity of rodents that move fruits of chica to their caves for storing (Campos et al. 2013; Zapata et al. 2017). These factors might be involved in the spatial genetic distribution observed in the analyzed genotypes of *R. girolae*.

Some genetic aspects of analyzed individuals of R. girolae

Contrary to our expectations, the level of genetic polymorphism detected by AFLP molecular markers within the analyzed natural population of *R. girolae* was high (Table 2). Since it is a monotypic species, it is not possible to compare the genetic diversity with other species of the same genus. Some examples performed in monotypic species with low restricted distribution revealed that genetic polymorphism is variable. Some examples of relatively high values of this parameter are described in other monotypic species of Fabaceae such as *Tamarindus indicus* (Pi=76.0%, AFLP marker) (Kumar et al. 2015) and *Plathymenia reticulata* (70.8%, RAPD marker) (Lacerda et al. 2001). However, another highly threatened species shows low values of genetic polymorphism. This is the case of some restricted populations of *Legendaria concinna* (Pi ranged among 22 and 56%) (Martínez Araneda et al. 2011). Furthermore, the comparison of our results with other species of trees and herbaceous legumes revealed contrasting genetic polymorphism. This is the case of *Acacia* sp. (Pj=19.1%, RAPD marker) (Casiva et al. 2002), *Prosopis* sp. (Pj=29.3%, RAPD marker) (Juárez-Muñoz et al. 2002), *Dalbergia nigra* (Pj=39.0%, RAPD marker) (Juchum et al. 2007), *Inga thibaudiana* (Pj=43.3%, RAPD marker) (Schierenbeck et al. 1997). Bérgamo de Souza and collaborators (2013) associated the loss of genetic diversity in *Parapiptadenia rigida* with the high level of deforestation in their natural environment (Pj=60.5%, AFLP marker). However, in the analyzed population of *R. girolae*, our results indicated that a wide and diverse genetic base exists. This seems to be a trait that is determined by its ecology and adaptation to hard climatic desert conditions.

Species with restricted geographic distribution generally have lower levels of genetic diversity than species with wide distribution (Hamrick and Godt 1989; Ribeiro et al. 2005). Unfortunately, most studies of tree and herbaceous legumes were made using different measurements of genetic diversity, hindering the comparison of results. However, the high level of genetic variability represented by the high percentage of polymorphic loci is consistent with the wide range of genetic diversity of Nei (He), independent of molecular marker employed. In the case of *R. girolae*, the analyzed population showed slightly higher mean values than the genetic variation values obtained in studies examining levels of genetic diversity between species of narrow range (He=0.215) and wide range distribution (He=0.267) (Hamrick and Godt 1989; Ribeiro et al. 2005). However, there have also been high levels of genetic variation records in other threatened tree legume species such as *Dalbergia nigra* (Papilonoideae) (Ribeiro et al. 2005) and *Caesalpina echinata* (Caesalpinioideae) (Cardoso et al. 1998). Similar results were found in species with restricted endemism belonging to other plant families such as *Seseli farrenyi* (Apiaceae) (López-Pujol et al. 2002), *Cochlearia bavarica* (Brassicaceae) (Paschke et al. 2002), *Colombobalanus excelsa* (Fagaceae) (Aguirre Acosta et al. 2013), *Vateriopsis seychellarum* (Dipterocarpaceae) (Finger et al. 2012) and *Antirhea aromatica* (Rubiaceae) (González-Astorga and Castillo-Campos 2004). The high genetic diversity detected in the analyzed *R. girolae* population might be due to numerous ecological factors such as low disturbance, presence of a high number of pollinator and seed-dispersers (Campos et al. 2017; Zapata et al. 2017).

Significant spatial genetic structure was detected in *R. girolae* using Mantel test and spatial autocorrelation. These results suggest

that our analyzed individuals of *R. girolae* exhibit a genetic spatial structure. Similar results were reported in other legume trees such as *B. monosperma* (Vashishtha et al. 2013) and *C. echinata* (Cardoso et al. 1998). Loss of SGS in woody species was associated with habitat fragmentation and deforestation (Bérgamo de Souza 2013; Piotti et al. 2013; Yineger et al. 2014). It is necessary to conduct more genetic studies of populations of *R. girolae* located in other areas of endemism and outside protected areas under different disturbances to conclude the conservation status of this species.

Conclusions

The genetic diversity of the studied *R. girolae* population was found to be high, thus highlighting the high conservation value of this site as a genetic pool. The calculated indexes suggest that there is an important contribution of genetic variability resulting from sexual reproduction. Nearby plants are closely related, and no clones were found. On the other hand, we confirmed that plants belonging to the same patch corresponded to the same genotype. Moreover, the autocorrelation analyses revealed a significant correlation between genetic and geographic distances, suggesting the existence of SGS. These results constitute the first exploratory analysis to the knowledge of the genetics of this endangered tree, endemic to the Monte of mountains and basins ecoregion. Finally, it is important to emphasize that the adjustment of the methodology used in this research represents a significant contribution to carry out similar studies in other natural populations of the species.

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