

# Population genetics of the Federally Threatened Miccosukee gooseberry (*Ribes echinellum*), an endemic North American species

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**Abstract** *Ribes echinellum* (Coville) Rehder (Miccosukee gooseberry; Grossulariaceae) is a Federally Threatened species known from only two localities: Jefferson County (Florida, FL) and McCormick County (South Carolina, SC). This perennial shrub, ca. 1 m tall, is deciduous, and reproduces both vegetatively (clonal growth) and sexually (seed production). Recent surveys of the FL population revealed a dramatic decline in plant numbers. To assist in conservation and management of this species in FL and SC populations, microsatellite genetic markers were used to identify genotypes and assess the genetic structure of *R. echinellum*. We genotyped seven microsatellite loci in 102 individuals: 74 collected in FL and 28 in SC. Unbiased heterozygosity was between 0.28 and 0.53. All seven loci were polymorphic, showing a range of 1.52–2.13 effective

number of alleles per locus (mean = 1.75). The two populations of *R. echinellum* show low genetic diversity, especially in SC. Clonality was not widespread, but was higher in the SC population. Both populations show signatures of bottlenecks but isolation by distance was not evident. We found significant deviation from HW equilibrium, with higher number of heterozygotes than expected. However when HW test was done for the combined populations as two separate groups, only FL showed a significant HW test and for SC the test was non-significant. Bayesian analysis and  $F_{ST}$  values suggest high genetic divergence between the populations. These results are important for developing a recovery plan and an ex situ and reintroduction conservation programs.

**Keywords** Population genetics · Miccosukee gooseberry · Federally Threatened · Microsatellites · Conservation · Florida and South Carolina

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## Introduction

Knowledge of a species' genetics can inform conservation and restoration initiatives in order to minimize the extinction risk by maintaining genetic diversity (Reed and Frankham 2003; Kramer and Havens 2009). Conservation of species with small and isolated populations are important because these species are expected to lose genetic variation over time which increases risk of extinction (Ouborg et al. 2006). Small populations also might experience an increase of inbreeding, which can result in poor growth, poor seed production, or low seed viability as a result of inbreeding depression (Ellstrand and Elam 1993). A metanalysis with plant species showed a significant positive correlation between population size, fitness and

genetic diversity (Leimu et al. 2006). Thus low genetic diversity might reduce the ability of the species to adapt with a changing environment (Jump et al. 2009; Markert et al. 2010). Furthermore genetic diversity within a population will influence important ecological aspects such as the ability of a population to recover from disturbance, interspecific competition, growth and stability of the population (Hughes et al. 2008). Neutral molecular markers can indirectly estimate mating patterns and genetic connectivity, which is valuable information for development of effective conservation and recovery practices (Rossetto and Rymer 2013).

*Ribes echinellum* (Coville) Rehder (Micosukee gooseberry; Grossulariaceae) is an extremely rare species globally classified as Critically Imperiled (G1) (NatureServe 2012), and designated as threatened in the Federal Register, July 18, 1985, (designation effective August 19, 1985; 50 FR 29338-29340) (Negrón-Ortiz 2007). The species was assigned a recovery priority number of 11, which indicates a species with moderate threat of extinction due to the presence of herbivory and several invasive species, but with low recovery potential as propagation efforts to date have proven unsuccessful and recovery rates appear slow. This species has only two small extant populations. In Florida it is located on private lands along the north shoreline of Lake Micosukee near Monticello, and in two public locations in McCormick County, South Carolina (Catling et al. 1998). Plants appear abundant in both locations, yet recent surveys of the FL population revealed a dramatic decline in plant numbers (Negrón-Ortiz 2007, Negrón-Ortiz 2008–2010 survey pers. obs.). Thus, the extremely constrained distribution of this species, and the small size and number of populations increases the probability of significant impacts from any losses (even small-scale perturbations), whether natural or from human impact.

*Ribes echinellum* is a perennial shrub ca. 1 m tall. It is deciduous, shedding most leaves after mid-summer with new leaves emerging in the autumn. The main floral visitors are two bees, *Bombus impatiens* and *Habropoda laboriosa*, for both FL and SC populations (Catling et al. 1998) (Fig. 1). *R. echinellum* reproduces both by clonal growth and seed production. Although seeds are produced, seedling recruitment has not been observed (Negrón-Ortiz pers. obs.). Based on this information, it is important to determine the genetic composition of *R. echinellum* populations to assist in conservation and management of this species in FL and SC. Our specific objectives in this study were to (1) assess genetic diversity of *R. echinellum* across the species geographic range, (2) estimate the degree of clonality in the species, (3) identify demographic patterns, and (4) to evaluate the degree of genetic connectivity between the two extant populations.



**Fig. 1** *Ribes echinellum* visited by a bumblebee

## Materials and methods

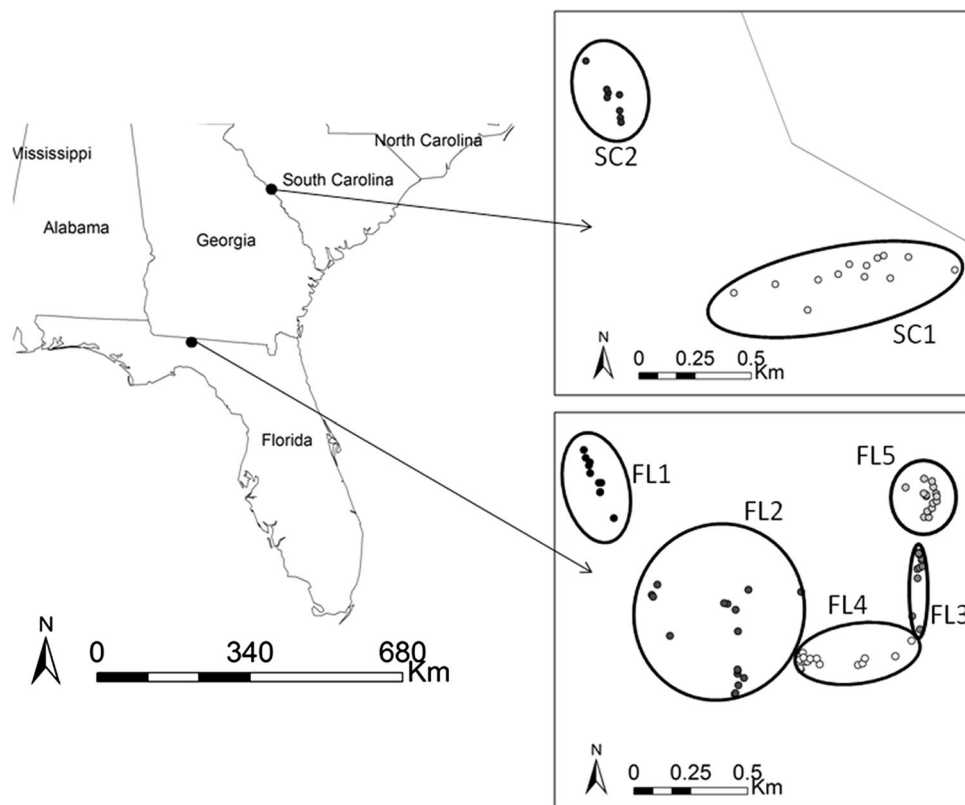
### Study sites and sampling procedure

Field searches were conducted across known localities of *R. echinellum* during Winter 2011, when most of its leaves were green. Young mature leaf material was sampled from 102 individuals collected across the geographical distribution of *R. echinellum* (Fig. 2). A complete-detailed field search was conducted within the north FL *R. echinellum* population (Fig. 2). These areas were monitored for *R. echinellum*, and the samples collected were geo-referenced to allow spatial analysis. Approximately 5–10<sup>2</sup> cm of leaf tissue was collected from each plant and kept chilled during transport to the University of FL.

### DNA isolations, microsatellite amplifications, and ABI analysis

DNA was extracted from leaves of 102 individuals (74 individuals from Jefferson County, FL and 28 individuals from McCormick County, SC) using QIAGEN kits (Valencia, CA, USA). Twelve samples were PCR amplified using each primer set originally designed of *Ribes nigrum*, which are available at <http://www.fruitbreeding.co.uk/RibesGenomics.asp>. Seven sets of primers proved to be polymorphic (e1-O01, e3-M04, e1-O21, g1-M07, e1-201, e3-B02, g1A01, g1-M07, g2-j08) and were used for the genetic analyses. Samples were multiplexed into a single lane for analysis on an AB3730xl (96 capillary automated sequencer). Amplicons were sized with LIZ600 and scored using GENEMARKER following well-established protocols in the manual (GeneMarker v. 1.7, Soft Genetics).

**Fig. 2** Collecting sites along the geographic distribution of *Ribes echinellum*



### Statistical analysis

Descriptive population genetic statistics (number of alleles per locus, number of private alleles, total heterozygosity, observed heterozygosity, fixation index and F-statistics) and analysis of molecular variance were estimated with GENALEX 6.3 (Peakall and Smouse 2006). Global HW test was calculated in GENEPOP (Rousset 2008). The degree of clonality was calculated as the genotype discovery rate ( $G/N$ ), which is the ratio between genotypes ( $G$ ) and number of individuals sampled ( $N$ ) (Peakall and Smouse 2006; Ellstrand and Rose 1987).

Spatial genetic structure (SGS) was assessed with autocorrelation analysis. We use the multilocus genetic correlation coefficient ( $r$ , Smouse and Peakall 1999). We divided our sample in 10 distances classes. A positive  $r$  means that individuals within the size class have more alleles in common than anticipated by chance (Smouse and Peakall 1999). Statistical significance of  $r$  was evaluated by comparing observed  $r$  values with 95 % confidence intervals obtained by 10,000 random permutations under the null hypothesis of no spatial genetic structure. Analysis of SGS was done with GENALEX 6.3 (Peakall and Smouse 2006).

Assignment of individuals to populations was performed with the program STRUCTURE 2.3.3. (Pritchard et al. 2000), in BIOPORTAL (Kumar et al. 2009). Our analysis was made under the admixture model, with 1,000,000

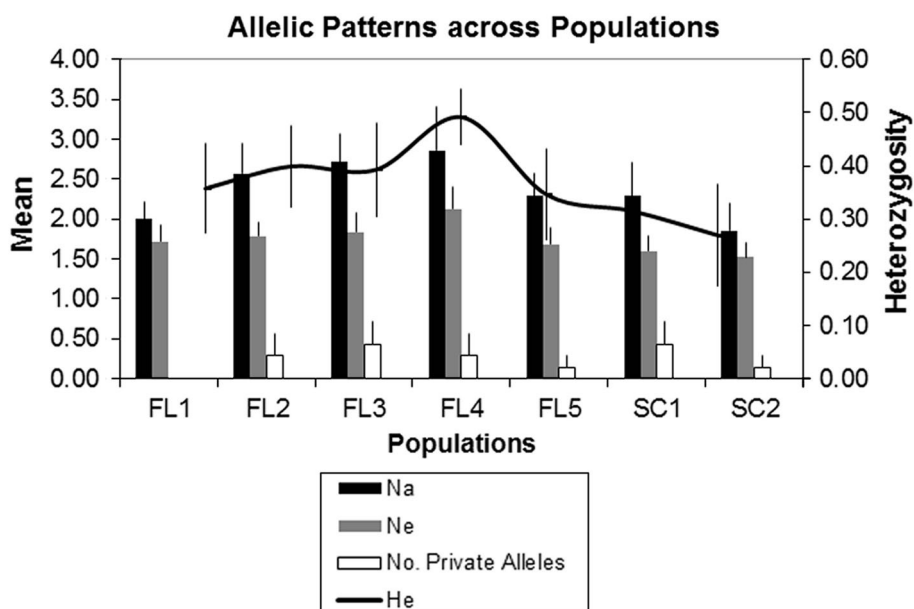
repetitions after a burn-in of 500,000 and it was replicated 20 times. We simulated this analysis for each  $k$  value (number of populations) ranging from 1 to 10. The results were uploaded into STRUCTURE HARVESTER (Earl 2011), which estimates the most likely  $k$  value, following Evanno et al. (2005) method. We used CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) to make a consensus of the results of the independent runs for the optimal  $k$ . For the consensus we use the Greedy option with random input order and 100,000 repeats. The consensus was visualized in DISTRUCT 1.1 (Rosenberg 2004). To determine if populations bore the signature of bottlenecks, we calculated use the heterozygosity excess approach in BOTTLENECK (Cornuet and Luikart 1996; Piry et al. 1999).

## Results

### Genetic diversity

Overall the genetic diversity of *R. echinellum* was low. All seven loci were polymorphic, with a range of 1.52–2.13 effective number of alleles per locus (mean = 1.75). Number of alleles, effective allele numbers, number of private alleles and heterozygosity were generally low, but consistently lower in SC (Fig. 3; Table 1). In general we found significant deviation from HW equilibrium, with

**Fig. 3** Allelic patterns across populations of *Ribes echinellum*. Allelic number ( $N_a$ ), effective allelic number ( $N_e$ ), heterozygosity ( $H_e$ )



**Table 1** Genetic diversity estimates in *Ribes echinellum* populations based on seven microsatellite loci

Region	Subpopulation	$N$	$N_a$	$N_e$	$G$	$G/N$	$MLG$	$H_o$	$U H_e$	$F$	$PA$	Wilcoxon test	
												IAM	SMM
Florida	FL1	10	2.00	1.72	10	1.00	0	0.51	0.38	-0.40***	0	0.04	0.22
	FL2	19	2.53	1.79	19	1.00	0	0.47	0.41	-0.16**	2	0.04	0.34
	FL3	13	2.71	1.84	11	0.85	2	0.59	0.41	-0.44***	3	0.28	0.58
	FL4	16	2.86	2.12	16	1.00	0	0.58	0.53	-0.18***	3	0.01	0.19
	FL5	16	2.29	1.68	15	0.94	1	0.43	0.36	-0.24 ns	1	0.08	0.42
South Carolina	SC1	13	2.29	1.60	11	0.85	2	0.34	0.34	-0.05 ns	3	0.40	0.69
	SC2	15	1.86	1.52	8	0.53	2	0.41	0.28	-0.52***	1	0.06	0.09
	Total Mean	12.35	2.34	1.75	11.89	0.84	1	0.47	0.39	-0.28	2.17		

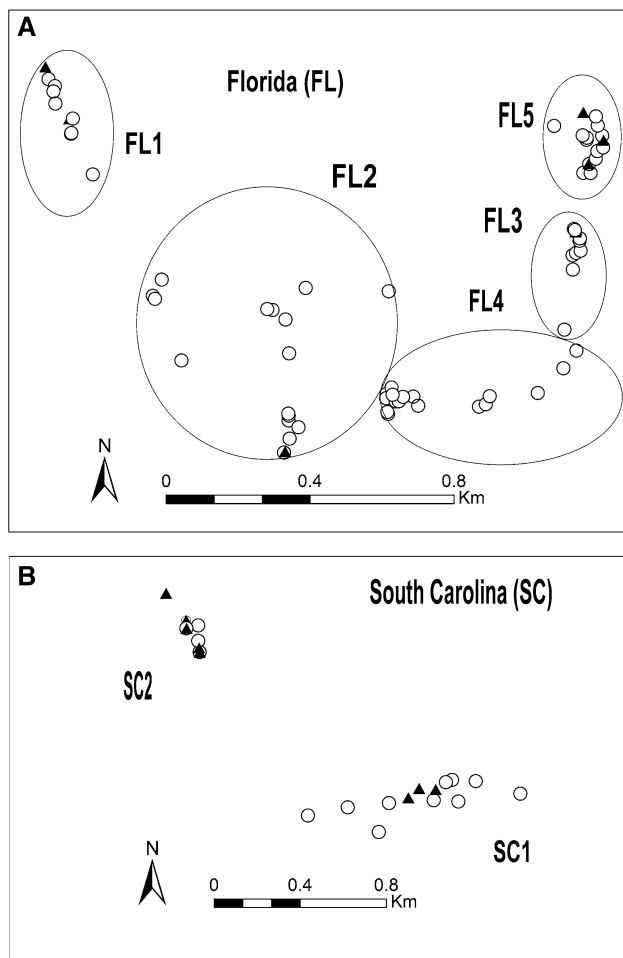
$N$  individual sample,  $N_a$  allele number,  $N_e$  effective allele number,  $G$  number of genets,  $G/N$  genotype discovery rate,  $MLG$  repetitive multilocus genotypes,  $H_o$  observed heterozygosity,  $H_e$  expected heterozygosity,  $U H_e$  unbiased expected heterozygosity,  $PA$  private alleles,  $IAM$  infinite allele model,  $SMM$  stepwise mutation model,  $ns$  non-significant

\*\*  $p < 0.05$ ; \*\*\*  $p < 0.001$

higher number of heterozygotes than expected (Table 1). However when HW test was done for the combined populations as two separate groups, only FL showed a significant HW test and for SC the test was non-significant (FL:  $p = 0.00$ ; SC:  $p = 0.63$ ).

Genetic differentiation between populations is high ( $F_{ST} = 0.26$ ). The combined data set resulted in 88 genets out of 102 samples. The number of individuals showing the same multilocus genotype (genets) varied from zero to five in each subpopulation (Table 1). We found 69 different genotypes for FL and 19 genotypes for SC. Values of the  $G/N$  were in the range of 1–0.53 (Table 1). We identified 81 unique multilocus genotypes. In each population the

number of putative clones was not equal. A total of 9 individuals out of 81 in FL were putative clones (Fig. 4a). For instance, in FL1 we found two individuals sharing the same genotype, whereas in FL2 one individual share the same genotype as another individual of FL1. In FL3 there were three individuals sharing the same genotype. On the contrary, FL4 did not have individuals with the same genotype. We found two individuals sharing the same genotype in FL5 and another individual share the genotype with one in FL1. On the other hand, we found more evidence of clones in SC populations, with three individuals having the same genotype in SC1 and a total of nine individuals being putative clones in SC2 (Fig. 4b).



**Fig. 4** Putative clones in *Ribes echinellum* populations. Open circles represent individuals with unique genotypes, whereas black triangles represent individuals showing a repetitive multilocus genotype. **a** Florida, **b** South Carolina

We found evidence of recent population reduction or bottleneck for Populations FL1, FL2 and FL4 only under infinite allele model (IAM) (Table 1). We did not find evidence of recent bottleneck for the SC populations using the heterozygosity excess approach.

**Spatial analysis and genetic structure**

Analysis of molecular variance shows significant geographic differentiation, the majority of the variance is within populations (65 %), followed by the variance among regions (24 %) and among populations (11 %) (Table 2). Bayesian assignment of individuals with STRUCTURE showed that almost all individuals were not admixed. Based on Evanno et al. (2005) method, we found two genetic clusters; one corresponds to the FL populations and the other to the SC populations (Fig. 5). We found weak isolation by distance (FL:  $R_2 = 0.22$ ,  $p = 0.0$ ; SC:  $R_2 = 0.39$ ,  $p = 0.0$ ).

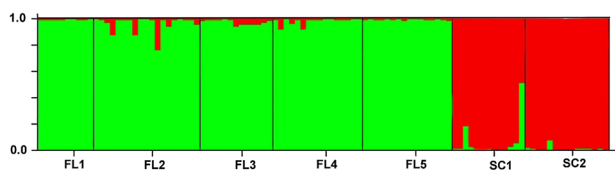
**Table 2** Analysis of molecular variance (AMOVA)

Source	df	SS	MS	Est. Var.	%	<i>p</i> value
Among regions	1	60.111	60.111	0.616	24	0.001***
Among populations	5	50.599	10.120	0.292	11	0.001***
Within populations	197	324.638	1.648	1.648	64	0.001***
Total	203	435.348		2.556	100	

Two hierarchical levels were analyzed: among regions (FL and SC) and at the population level

*Df* degree of freedom, *SS* sum of squares

\*\*\* Significant



**Fig. 5** Population genetic structure of *Ribes echinellum* inferred by Bayesian analysis. Plot bar of the estimated population structure per each of the species' populations  $k = 2$ . Colors in each vertical bar represent the individual ancestry  $q$ . Florida (FL), South Carolina (SC)

However, we found a significant positive spatial autocorrelation across distances <500 m (Fig. 6).

**Discussion**

**Genetic diversity**

The populations of *R. echinellum* analyzed in this study show low genetic diversity, especially in SC. No evidence of strong clonality was found; however, in SC putative clonality was higher and especially high in SC2. The subpopulation SC2 is represented by one large cluster and seven small subgroups (each 1–2 m<sup>2</sup>), which might explain the clonality results. We also found evidence of bottlenecks in FL population, which means that in the recent past the FL populations were subject to a reduction in the number of individuals. Surprisingly, the SC populations did not show evidence of bottleneck with the heterozygote excess approach. This result could be related to lack of power because of a small sample size (less than 20 individuals) and a relatively low number of loci used in our study (Cornuet and Luikart 1996). Another possible explanation for the low genetic diversity but lack of recent bottleneck evidence in the SC population may be related to Pleistocene refugia; this population might be a remnant of the species distribution during climate change periods (James



1961). The excess of heterozygotes in microsatellite loci found in *R. echinellum* is consistent with heterozygous overdominance. Selection for heterozygous overdominance in perennial species can be the response to a shift towards individual survival to the detriment of population reproductive fitness (Wiens and Slaton 2012). High number of heterozygotes was found also in another microsatellite study in *Ziziphus celata*, an endangered shrub endemic to FL (Gitzendanner et al. 2012), however *Z. celata* was highly clonal, which was not the case of *R. echinellum*.

Comparing the genetic structure of the two disjoint populations, Bayesian cluster analysis and  $F_{ST}$  values suggest high differentiation between the FL and SC populations. Also, genetic variation in *R. echinellum* is spatially autocorrelated and our results indicate that there is a differentiation at longer distances (>500 m). The strong genetic differentiation among regions shows that there is a lack of connectivity by pollination and seed dispersion between FL and SC, separated by ca. 350 km and with no continuous habitat in between. In summary the low diversity, the signature of recent population bottlenecks found in this study and the lack of seedling recruitment for *R. echinellum* (Negrón-Ortiz, pers. obs.) suggest significant genetic peril for this species.

Our results should be used as a starting point to better understand the genetic implications towards the conservation of *R. echinellum*. Our sampling only used adult individuals to minimize disturbance and because they are nearly all of the extant individuals in these populations. Also using mature individuals will show the genetic signal of past demographic change. The genetic signature found in our study shows solely information on adult individuals, which could be different for seedlings. Genetic studies of seedlings will provide information about current conditions affecting the genetic variation of the species. Future work can track changes in demography and genetic structure with genetic surveys performed in subsequent years.

### Conservation implications

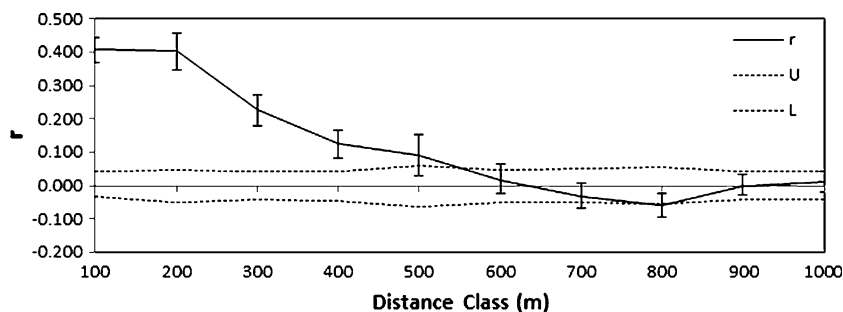
Given the low levels of genetic variation, our findings underscore the importance of protecting *R. echinellum*

through both in situ and ex situ conservation strategies. According to the last evaluation of *R. echinellum* report, the species' recovery potential is low and seed recruitment appears to be rare (Negrón-Ortiz, pers. obs. 2007–2011). Seed viability is approximately 60 % (Negrón-Ortiz, unpublished). Selection for heterozygous overdominance might explain the low reproductive success we have observed. High levels of heterozygosity have been found in several long-lived plants in environmentally stressful conditions when heterosis might be beneficial for survival (Wiens and Slaton 2012). At the same time, high rates of embryonic abortion in the aforementioned plants might be the result of the increase of deleterious alleles, and as a result it could produce a decrease of fecundity (Wiens and Slaton 2012). For expansion initiatives, artificial crosses from individuals at greater spatial distances or with different microsatellite profiles can be made under in situ conditions. Crossings aim to produce heterozygotes that may improve seed set if few self-incompatibility alleles are present in close proximity in the remaining populations. With regard to ex situ conservation, germplasm should be separately collected from the two different populations, as the Bayesian genetic structure indicates that the FL and the SC represent different genetic clusters. Within populations, cuttings should be obtained from individuals located at greater spatial distances.

Based on our analyses, seeds from subpopulations FL3 and FL4 should be chosen for ex situ initiatives because both are the localities with the highest genetic diversity. On the other hand the SC need urgent conservation action such as because both subpopulation and specially SC2 show the lowest genetic diversity of the species.

All subpopulation within regions showed low effective number of alleles, but FL populations had higher number of alleles. Specifically subpopulation FL 4 shows higher level of genetic diversity and the individuals of this group might be considered good candidates for augmentation or reintroduction programs. The most genetically diverse populations located in FL are also privately owned, which will require fostering a working partnership with the landowners to preserve *R. echinellum*. To enhance seed production, we recommend hand pollination experiments to determine whether heterosis or biparental inbreeding may be occurring and responsible for the low or lack of seed germination and

**Fig. 6** Analysis of spatial genetic structure of *Ribes echinellum*. Correlogram plot of the genetic autocorrelation coefficient as a function of the geographic distance, dotted lines are the 95 % coefficient interval based on 999 permutations



seedling recruitment in the FL population. Overall, we recommend continued conservation actions to address the lack of seedling recruitment in the extant populations.

*Ribes echinellum* faces several other threats. Despite the fact that *R. echinellum* from SC is located in public areas acquired by the SC Department of Natural Resources, deer browse, and more recently feral hogs, could be a cause for the decline observed in this population (Negrón-Ortiz 2007). In the FL population, deer are also a threat, as is encroachment of unprotected portions of the population by development. Moreover invasive species like the Chinese privet (*Ligustrum sinense*) (Langeland and Burks 1998) might displace *R. echinellum*.

The results of this study provide information of the current genetic diversity in the two natural remaining populations of *R. echinellum*. Our results highlight the need for increased conservation management for this species. Restoration programs will benefit from the current information because it constitutes a genetic assessment baseline. The identified genets should be further used as pollen donor/recipients to test whether cross-pollinating genets can produce viable seeds. Secondly, genetic diversity in seedlings and in newly established populations can be compared to our results in order to ensure that genetic diversity is at least maintained at the current levels. Genetic diversity should be monitored to identify potential looming threats such as inbreeding depression.

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