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RESEARCH ARTICLE

Assessing genetic diversity for the USA endemic carnivorous plant *Pinguicula ionantha* R.K. Godfrey (Lentibulariaceae)

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Abstract Understanding patterns of genetic diversity and population structure for rare, narrowly endemic plant species, such as *Pinguicula ionantha* (Godfrey's butterwort; Lentibulariaceae), informs conservation goals and can directly affect management decisions. *Pinguicula ionantha* is a federally listed species endemic to the Florida Panhandle in the southeastern USA. The main goal of our study was to assess patterns of genetic diversity and structure in 17 *P. ionantha* populations, and to determine if diversity is associated with geographic location or population characteristics. We scored 240 individuals at a total of 899 AFLP markers (893 polymorphic markers). We found no relationship between the estimated population size with either of two measures of diversity (proportion of loci polymorphic, $P = 0.37$; Nei's gene diversity, $P = 0.50$). We also found low levels of population genetic structure; there was no clear relationship of genetic isolation by distance ($P = 0.23$) and only a small (but significant) proportion of genetic variation was partitioned amongst regions (2.4 %, $P = 0.02$) or populations (20.8 %, $P < 0.001$). STRUCTURE analysis found that the model with two inferred clusters ($K = 2$) best described the AFLP data; the dominant cluster at each site corresponded to the results from PCoA and Nei's genetic distance analyses. The observed patterns of genetic diversity suggest that although *P. ionantha* populations are isolated spatially by distance and both natural and anthropogenic barriers, some gene flow occurs among them or isolation has been too recent to leave a genetic signature. The relatively low level of genetic diversity associated with this species is a concern as it may impair fitness and evolutionary capability in a changing environment. The results of this study provide the foundation for the development of management practices that will assist in the protection of this rare carnivorous plant.

Keywords *Pinguicula ionantha* · AFLP · Conservation genetics · Endemic · Carnivorous plant · Rare species biology

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Introduction

Characterization of genetic diversity and structure in rare plants is often necessary for effective conservation because it provides insight into important aspects of demography, reproduction, and ecology. Narrowly endemic species, due to their rarity, are susceptible to low genetic diversity and its negative consequences (Gitzendanner and Soltis 2000), such as inbreeding depression and loss of evolutionary potential. Additionally, narrowly endemic species are often restricted to rare habitats that may be under pressure from anthropogenic disruption, which can further accelerate loss of genetic variability (Karron 1987; López-Pujol et al. 2013). Information on genetic patterns can predict the effectiveness of common management strategies, and how best to carry out those strategies. For example, information

on patterns of genetic diversity can determine which populations are genetically depauperate (Gitzendanner et al. 2012; Backs et al. 2015), and which other populations may be appropriate sources for augmentation or reinforcement (Demauro 1993; Tecic et al. 1998; Kim et al. 2015). Additionally, insights into natural patterns of gene flow and diversity can lay the baseline for recovery goals.

Worldwide it is estimated that more than 600 carnivorous plant species from 17 genera exist (Ellison and Gotelli 2009), with many of these considered threatened (Jennings and Rohr 2011). The large proportion of vulnerable carnivorous plants—due to habitat loss, over-collection, pollution, and other factors (Jennings and Rohr 2011)—makes the group especially well suited to studies of conservation genetics. The genus *Pinguicula* (butterworts, Lentibulariaceae) is an important group of carnivorous plants that is often overlooked with respect to conservation genetics, with more attention focused on carnivorous plants in the genera *Sarracenia* and *Drosera* (e.g., Koopman and Carstens 2010; Chung et al. 2013; Furches et al. 2013; but see Alcalá and Domínguez 2012). The genus *Pinguicula* is comprised of plants found in all continents except Australia (Blanca et al. 1999; Legendre 2000; Heslop-Harrison 2004). *Pinguicula* species have solitary flowers and leaves in a basal rosette. Fruits are dry capsules with small brown seeds. *Pinguicula* leaves produce a secretion that captures and digests mostly small arthropods. Based on taxonomic (e.g., Zamora et al. 1996; Conti and Peruzzi 2006; Yildirim et al. 2012) and phylogenetic studies (e.g., Cieslak et al. 2005) more than 100 species have been identified in the genus (Rodondi et al. 2010). Six species of *Pinguicula* are found in the southeastern USA, and all but one of these six species is considered rare (Wunderlin and Hansen 2008).

Pinguicula ionantha R.K. Godfrey (Godfrey's butterwort) is endemic to the state of Florida in the southeastern USA. This species is only found in six counties within the northwestern portion of Florida known as the Panhandle (Fig. 1). It was listed as federally threatened in 1993 due to habitat loss and degradation (i.e., logging, urban development, and fire suppression; U.S. Fish and Wildlife Service 2009). NatureServe has assigned the species a Conservation Status Rank of G2-Imperiled (NatureServe 2015). It is a short-lived perennial found in moist to wet habitats ranging from shrub bog-savannas, drainage ditches, and depressions within pine flatwoods (Godfrey and Stripling 1961; U.S. Fish and Wildlife Service 2009). The solitary flowers are found on long peduncles rising from a basal rosette. Flowers are lavender to white with a tubular corolla that ends in a yellow to olive spur. The corolla throat and tube are dark purple with purple veins. The primary pollinators are bees (B. Molano-Flores, personal observation). After fertilization, flowers develop into a dry capsule with numerous small brown seeds. Seeds can be dispersed by water after the capsules dehisce (B. Molano-Flores, personal observation). Although information about many aspects of the biology and ecology of the species are known [i.e., elements of occurrence, habitat characteristics, chromosome number, demography, digestive mechanism, and phylogenetic relationships within the genus; (Godfrey and Stripling 1961; Heslop-Harrison AND Heslop-Harrison 1981; Cieslak et al. 2005; Kesler et al. 2008; Casper and Stimper 2009; U.S. Fish and Wildlife Service 2009)], no researchers have studied the population genetics of *P. ionantha*. Because the remaining populations are isolated and many are small, it is important to determine the relative diversity of extant populations, whether low diversity is associated with population size, isolation, or performance, and what action would best suit populations that may be in need of management.

The main goal of this study was to characterize the population genetics of *Pinguicula ionantha.* In particular we aim to determine: (1) how genetic diversity (proportion of polymorphic loci, Nei's gene diversity) is apportioned within and among sites, (2) if estimates of population size are associated with the levels of diversity, (3) whether genetic structure exists among our 17 study sites, (4) how the physical distance and geographical location of study sites are related to genetic structure and genetic distance. These results can be utilized to rank the demes of *P. ionantha* at each site in terms of their genetic vulnerability and identify where management is most needed, and our findings can be combined with other knowledge of the species's biology to develop strategies for in situ and ex situ conservation.

Materials and methods

A total of 240 individuals from 17 sites of *Pinguicula ionantha* were sampled (Table 1; Fig. 1), including four of the six counties from which *P. ionantha* is known to occur. We sampled individuals from six sites in Liberty County (L1–L6), four sites in Franklin County (F1–F4), two sites in Gulf County (G1–G2), and five sites in Bay County (B1–B5). Two sites with large estimated population sizes (L2 and B4) had small sample sizes for genetic analysis because a large proportion of the individuals observed at the time of sampling were seedlings that were not suitable for leaf collection.

DNA was extracted from dried leaf tissue using a Qiagen DNeasy Kit (Qiagen, Germantown, MD). Amplified Fragment Length Polymorphism (AFLP) molecular markers were used to assess patterns of variation within and between the populations of *P. ionantha.* AFLPs are dominantly inherited genetic markers that are useful in population-level studies because they provide a high number of

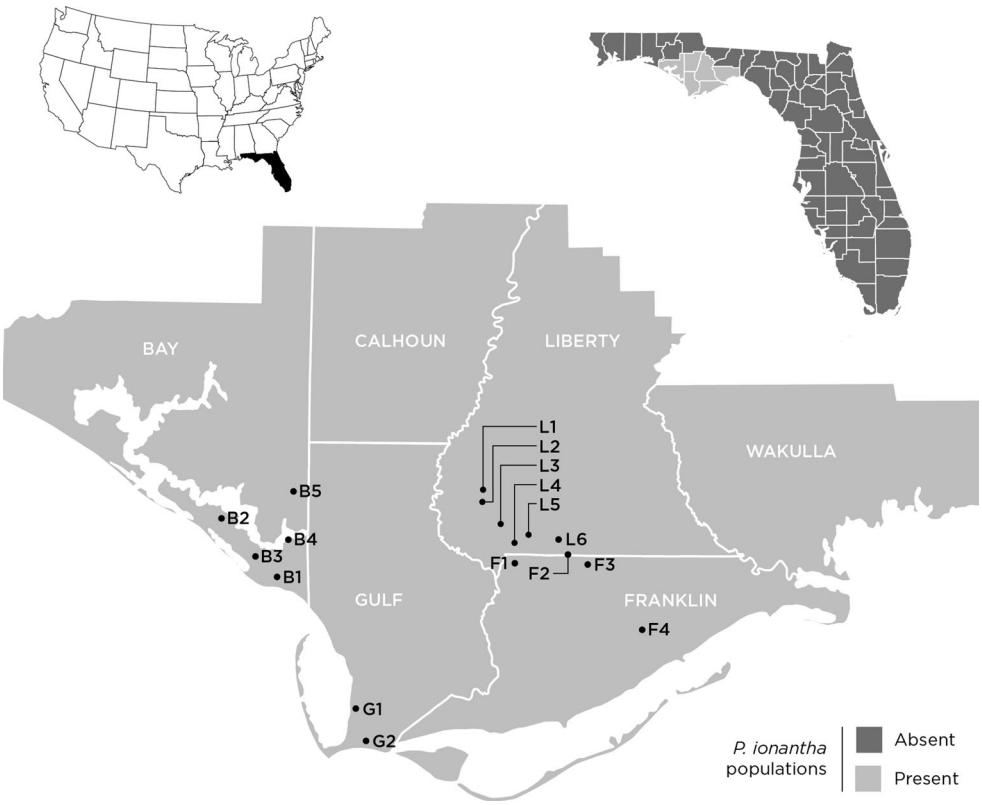

Fig. 1 Location of 17 sampling sites of *Pinguicula ionantha* in Florida, USA. The species is known to occur in six counties in the Florida Panhandle: Bay, Calhoun, Franklin, Gulf, Liberty, and Wakulla. We sampled individuals from four counties

Table 1 Population genetics for *Pinguicula ionantha* based on 893 polymorphic AFLP markers

Site	County	n	PLP (%)	H_j	Private alleles	Population size	Region
B1	Bay	15	23.9	0.08389	20	73[b]	West
B2	Bay	30	26.7	0.08372	27	326[b]	West
B3	Bay	8	33.6	0.10637	18	23[b]	West
B4	Bay	7	33.9	0.11177	18	97[a]	West
B5	Bay	2	2.8	0.01590	8	3[c]	West
F1	Franklin	15	8.0	0.03491	16	65[d]	East
F2	Franklin	10	38.4	0.10638	22	30[b]	East
F3	Franklin	11	25.3	0.10958	19	50[b]	East
F4	Franklin	5	29.1	0.11074	8	37[b]	East
G1	Gulf	8	7.2	0.01904	13	12[c]	South
G2	Gulf	30	28.4	0.09192	37	1022[c]	South
L1	Liberty	25	7.2	0.02945	16	67[d]	East
L2	Liberty	8	35.2	0.10165	23	126[b]	East
L3	Liberty	7	25.3	0.07896	6	30[b]	East
L4	Liberty	19	18.0	0.05157	29	115[b]	East
L5	Liberty	30	11.4	0.04175	18	340[d]	East
L6	Liberty	10	44.7	0.13030	27	92[d]	East

Region gives the classifications used in the analysis of molecular variance (AMOVA)

PLP proportion of polymorphic loci at the 5 % level (marker frequencies lying within the range of 0.05 to 0.95), expressed as a percentage; H_j Nei's gene diversity (analogous to H or H_e in most publications; Vekemans 2002); *n* sample size for genetic analysis.

Population size data: [a]average from 2005, 2008 and 2011; [b]2012; [c]2013; [d]2014

genetic markers used as "fingerprints" (e.g., Escaravage et al. 1998; Scariot et al. 2007; Falinska et al. 2010; de Witte et al. 2012). The great number of markers and coverage of the entire genome allows for fine resolution of population structure (Scariot et al. 2007).

Methods for AFLP analysis generally followed Vos et al. (1995) and a modified protocol of the Applied Biosystems Plant Genome kit developed by M. Gitzendanner (University of Florida; personal communication) with the following modifications: Genomic DNA, 2 μL was digested for 3 h at 37 °C with 0.15 μL of EcoRI-HF (20 U/μL; New England Biolabs, Beverly, MA), 0.25 μL MseI (10 U/μL; NEB), 1.0 μL the CutSmart enzyme buffer (NEB), to a final reaction volume of 10 μL. The digest reaction was stopped with a 65 °C soak for 20 min. A 10 μL ligation reaction containing 0.5 μL of T4 DNA ligase (3 U/μL; NEB), 2 μL 10X T4 Ligase buffer (NEB), 1.8 μL MseI adapter (50 μM; 5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3'), and 1.8 μL EcoRI adapter (5 μM; 5′-CTCGTAGACTGCGTACC-3′ and 5′-AATTGGTACGCAGTCTAC-3′) was then added to the completed restriction digest and run at 16 °C overnight followed by 10 min at 65 °C.

The first selective amplification was conducted in 20 μL reaction volumes containing 4 μL of the restriction-ligation reaction, 0.2 μL One*Taq* DNA polymerase (5 U/μL; NEB), 4 μL 5X Standard One*Taq* buffer (NEB), 1.6 μL 10 mM dNTPs, 1.2 μL EcoRI + 1A primer (5 μM; 5′-GACTGCGTACCAATTCA-3′), and 1.2 μL MseI + 1C primer (5 μM; 5′-GACGATGAGTCCTGAGTAAC-3′). Reactions were heated to 72 °C for 2 min, then cycled 20 times at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 120 s, and then held at 60 °C for 30 min. These reactions were diluted in 250 μL of sterile water and used in the second selective amplification step. The second amplification was performed in 15 μL reactions containing 3.75 μL diluted + 1 PCR product, 0.15 μL One*Taq* Hot Start DNA polymerase (5 U/μL; NEB), 3 μL 5X Standard One*Taq* Buffer (NEB), 1.2 μL 10 mM dNTPs, 0.45 μL of each EcoRI + 4 primer (1.65 μM each; 5′-[6-FAM] GACTGCGTACCAATTCACAT-3′; 5′-[NED] GACTGCGTACCAATTCAAGT-3′; 5′-[VIC] GACTGCGTACCAATTCAACT-3′; 5′-[PET] GACTGCGTACCAATTCACCT-3′), and 0.38 μL MseI + 4 primer (5 μM; 5′-GACGATGAGTCCTGAGTAACTCA-3′).

The +4 primers were chosen because of their increased selectivity, to avoid homoplasy (Althoff et al. 2007). The EcoRI + 4 primers were fluorescently labeled for visualization on an automated DNA analyzer. Single reactions contained four primer combinations because each primer was labeled with a different wavelength of dye (i.e., each EcoRI + 4 primer with the MseI + 4 primer). The reactions were held at 94 °C for 2 min, then cycled 10 times starting at 94 °C for 30 s, 65 °C for 30 s, 72 °C for 2 min, with a reduction in the annealing temperature by 1 °C per cycle. Reactions were then cycled 36 times at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min, followed by a 30-min 60 °C hold.

The +4 PCR reactions were sent to the Roy J. Carver Biotechnology Center, University of Illinois, Urbana, IL, to be run on an Applied BioSystems 3730xl DNA Analyzer. Data were analyzed with GeneMapper (v3.7; Applied Biosystems, Foster City, CA). The program's default settings were used with the following exceptions: only fragments from 100 to 500 base pairs (bp) were analyzed, the allele calling threshold was set to 50, common alleles were not deleted, and "advanced peak detected" was selected. Fragments smaller than 100 bp were removed from the study to avoid fragment-size homoplasy (Althoff et al. 2007). Semi-automated scoring was verified manually for a sample of individual chromatograms.

The fragments were analyzed with AFLP-SURV (Vekemans 2002) to calculate basic genetic diversity statistics (proportion loci polymorphic [PLP] and expected heterozygosity under Hardy–Weinberg genotypic proportions, or Nei's gene diversity [H_j]), population structure (F-statistics [F_{ST}]), and genetic similarity as Nei's genetic distance [D]).

A neighbor-joining analysis with genetic distance using PAUP* (Swofford 2002) was conducted to assess genetic similarity among the different sites. We analyzed AFLP data with the Bayesian clustering program STRUCTURE version 2.3.4 (Pritchard et al. 2000; Hubisz et al. 2009), to assess the degree of admixture within and between populations. The default settings were used for STRUCTURE (admixed ancestry model, correlated allele frequencies) because of the possibility that populations were genetically connected, at least in the recent past (Porras-Hurtado et al. 2013). The number of clusters tested ranged from $K = 1$ to 17, and data were collected from 3 runs of 50,000 MCMC repetitions (after a burnin period of 25,000; visual inspection showed that values of Alpha, F_{ST}, and Likelihood were stable after the burnin period). The optimal number of inferred clusters was chosen by maximizing ΔK using the method described by Evanno et al. (2005), implemented in the program Structure Harvester (Earl and Vonholdt 2012).

We used a Mantel test, implemented with the *vegan* package (Oksanen et al. 2014) in R version 3.1.2 (R Core Team 2014), to test for isolation by distance. The Mantel test was repeated using Pearson, Kendall, and Spearman correlations; we report the latter for conciseness, but results were similar across the three correlation techniques. The Mantel test utilized geographic distances calculated with the *fossil* package (Vavrek 2011) and Nei's genetic distances calculated with AFLP-SURV; using log-distances (data not presented) did not change our results. To assess how genetic variation was partitioned among regions,

among populations, and within populations we conducted an analysis of molecular variance (AMOVA) using the *pegas* package in R (Paradis 2010). Three regions, derived from the geographical location of the sites, were used in the AMOVA analysis: east, west, and south (Table 1). Both the R package *ade4* (Dray and Dufour 2007) and GenAlEx version 6.5 (Peakall and Smouse 2012) were independently used for principle coordinates analysis (PCoA) at the site level, to reduce the variation at all AFLP markers to a few composite axes (eigenvalues). The two tools yielded the same results. The first two eigenvalues were plotted to show similarity patterns among different sites.

In addition, we tested the relationship between the estimated population size and proportion of polymorphic loci as well as between log population size and Nei's gene diversity with Spearman's rank correlation tests. Multiple studies have shown that such relationships exist for many rare plants (Schmidt and Jensen 2000; Ilves et al. 2013). Population size data for *P. ionantha* from different sources were available to conduct these analyses (Table 1). Estimates of population size included all individuals (not just reproductive individuals). For most populations estimates come from censuses. For larger population sizes the density in a sample of the study area was quantified and the overall population size estimate was calculated through extrapolation.

Results

A total of 899 AFLP markers were scored for *P. ionantha*, including six monomorphic markers. When only considering the 893 polymorphic markers, the proportion of polymorphic loci (PLP) in *P. ionantha* populations varied from 2.8 % to 44.7 % (Table 1) with a mean of 23.5 % (standard error = 12.4 %). Nei's gene diversity (Hj) at the site level varied from 0.0159 to 0.1303 (Table 1) with a mean of 0.0769 (se = 0.009). The number of private alleles varied from 6 to 37 (Table 1) with a mean of 19.1 (se = 2.0). No significant correlations were found between population size and proportion of polymorphic loci (Spearman rho = 0.231, P = 0.372, n = 17) or between population size and Nei's gene diversity (Spearman rho = 0.177, P = 0.498, n = 17). Across all populations, $F_{ST} = 0.1384$.

Genetic distance (Nei's genetic distance [D]) among our study sites ranges from 0 to 0.0384 (Table 2). Pairwise geographic distances ranged from 1.1 to 81.5 km (mean, 34.9 km, se = 4.7 km, median, 39.7 km). F3 and F4, geographically separated by 16 km, are genetically the most similar (D < 0.0001), while B5 is the most different from a number of sites (D = 0.0384 compared to G2, which is 49 km away; D = 0.0382 compared to F2, 53 km away; D = 0.0374 compared to B1, 17 km away; and D = 0.0367 compared to B2, 15 km away). If one only includes sites with greater than 10 individuals sampled, D ranges from 0.001 to 0.0282 (L1 and F2, separated by 44 km). The PCoA (Fig. 2) and neighbor-joining algorithm based on genetic distance (not pictured) agreed that there are two distinct groups of sites. Populations L1, L4, L5, F5, B5 and G1 form one group, while all of the other populations form a second group (Fig. 2). The two most intermediate populations are L2 and L3. The genetic groupings do not match the geography, as sites in the eastern, western, and southern portions of our study area were placed in each genetic group (Table 1; Figs. 1, 2).

The STRUCTURE results found that $K = 2$ (number of inferred clusters) fit the data best, giving the greatest ΔK (Fig. 3). The six sites listed above as forming a single group in PCoA and neighbor-joining analyses (L1, L4, L5, F5, B5 and G1) were dominated by the first genetic cluster, while the other populations were dominated by the second genetic cluster (Table 3). The proportion of admixed individuals (maximum $q \leq 0.85$) in each of the two groups was similar (22.2 versus 19.9 %, Chi squared = 0.08, df = 1, P = 0.78).

A Wilcoxon rank sum test comparing genetic diversity between the two groups showed a significant difference in PLP (W = 0, P = 0.001) and Hj (W = 0, P = 0.0002). In both cases, group 2 had greater values (Fig. 2). Group 1 had 325 private alleles, and Group 2 only had 78 private alleles. However, the two groups did not differ significantly in population size (W = 28, P = 0.65) and genetic sample size (W = 40.5, P = 0.48).

There was no significant isolation by distance as tested with a Mantel test (based on either Pearson, Kendall, or Spearman correlations; P > 0.226, in all three cases; Fig. 4). Notably, some of the most distant population pairs had lowest genetic pairwise genetic distances (e.g. B3 and F4), while some of the nearest population pairs had the greatest genetic distances (e.g. G1 and G2; Figs. 1 and 4; Table 2). Furthermore, we conducted Mantel tests within the two genetic groups observed through STRUCTURE and the PCoA, and still did not find significant isolation by distance (P > 0.2 for both groups). In addition, AMOVA also showed a relative lack of genetic structure over larger geographic scales; for *P. ionantha* only 2.4 % of variation is among geographic regions (P = 0.022), 20.8 % of variation is among sites (P < 0.001), and 76.7 % of the variation is within sites (P < 0.001). These data indicate a moderate amount of gene flow in *P. ionantha*, but that connectivity does not (or did not) follow clear geographic patterns.

Discussion

Pinguicula ionantha is a rare endemic carnivorous plant found in the southeastern USA. The results of this study provide the first assessment of genetic diversity associated

Table 2 Nei's genetic distance [D] (after Lynch and Milligan 1994) for pairwise site comparisons of *Pinguicula ionantha*

	L5	L4	L1	L3	F1	B5	G1	G2	B1	B2	B3	L6	F3	F2	F4	B4
L2	0.0090	0.0076	0.0117	0.0004	0.0109	0.0205	0.0168	0.0069	0.0057	0.0068	0.0034	0.0056	0.0025	0.0051	0.0027	0.0018
L5	–	0.0012	0.0008	0.0086	0.0015	0.0039	0.0016	0.0237	0.0227	0.0218	0.0188	0.0189	0.0189	0.0242	0.0211	0.0194
L4	–	–	0.0020	0.0073	0.0025	0.0077	0.0037	0.0196	0.0192	0.0173	0.0161	0.0159	0.0155	0.0210	0.0188	0.0175
L1	–	–	–	0.0102	0.0005	0.0039	0.0015	0.0272	0.0262	0.0250	0.0212	0.0223	0.0225	0.0282	0.0247	0.0221
L3	–	–	–	–	0.0097	0.0173	0.0159	0.0070	0.0052	0.0063	0.0024	0.0070	0.0040	0.0056	0.0042	0.0043
F1	–	–	–	–	–	0.0050	0.0023	0.0256	0.0263	0.0249	0.0216	0.0215	0.0219	0.0271	0.0237	0.0204
B5	–	–	–	–	–	–	0.0025	*0.0384*	0.0374	0.0367	0.0291	0.0304	0.0324	0.0382	0.0336	0.0320
G1	–	–	–	–	–	–	–	0.0350	0.0341	0.0333	0.0281	0.0276	0.0293	0.0356	0.0308	0.0286
G2	–	–	–	–	–	–	–	–	0.0044	0.0026	0.0055	0.0060	0.0032	0.0028	0.0054	0.0072
B1	–	–	–	–	–	–	–	–	–	0.0021	0.0039	0.0095	0.0034	0.0040	0.0055	0.0054
B2	–	–	–	–	–	–	–	–	–	–	0.0057	0.0073	0.0032	0.0026	0.0046	0.0082
B3	–	–	–	–	–	–	–	–	–	–	–	0.0043	0.0019	0.0032	0.0003	0.0054
L6	–	–	–	–	–	–	–	–	–	–	–	–	0.0025	0.0031	0.0046	0.0087
F3	–	–	–	–	–	–	–	–	–	–	–	–	–	0.0001	*0.0000*	0.0046
F2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.0017	0.0061
F4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.0027
B4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Most sites are genetically similar

Lowest and highest values are italics

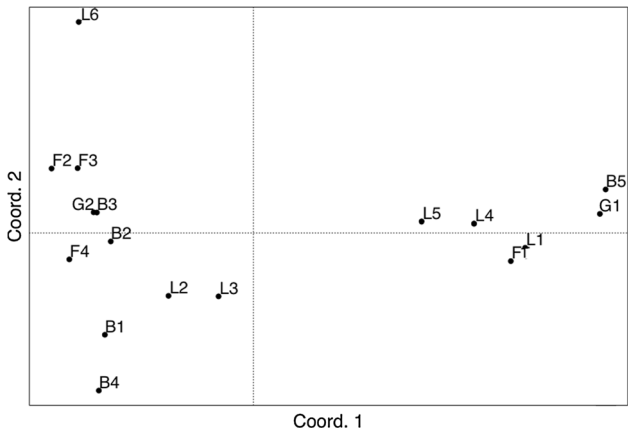

Fig. 2 Two populations clusters were identified by the PCA plot. The first PCoA axis explained 74.6 % and the second axis explained 6.8 % of the variance. Noteworthy is that both clusters have populations that are not found in geographic proximity (see Fig. 1)

with *Pinguicula* species in this region. Results show that populations of *P. ionantha* have varying levels of genetic diversity, though the proportion of polymorphic loci and Nei's gene diversity were generally low. We found no significant relationship between population size and the two measures of genetic diversity we calculated. In addition, although sampled sites were very similar to each other, some level of population structure was found. Sites could generally be classified into two groups, which significantly differed in the measures of genetic diversity. The population structure did not follow clear geographic patterns, as sites in the two groups were intermixed geographically, and we found no isolation by distance or strong regional differentiation. Additionally, admixture was observed at most sites. With the evidence available, we cannot determine whether the large differences between sites with respect to diversity and number of private alleles (Table 1) are due to natural factors or habitat loss and fragmentation, nor can we tell when the differences in these metrics was first established.

When compared to other studies that use AFLP or RAPD for plants, we found mean values of within-population genetic diversity and differentiation in *P. ionantha* that were approximately one-half of the means observed in other endemic species (Nybom 2004). Also, we found lower levels of genetic diversity and structure than in the only other comparable population genetic study of in *Pinguicula* that we are aware of, a study of four populations of *Pinguicula moranensis* (Alcalá and Domínguez 2012). One potential cause for the observed difference from *P. moranensis* is the difference in habitats and the resulting degree of isolation. *Pinguicula moranensis* has a mountainous distribution in central Mexico, leading to strong natural barriers to gene flow and setting the stage for greater differentiation (Alcalá and Domínguez 2012). Meanwhile, *P. ionantha* occurs in flatwoods that once dominated the Florida Panhandle until recent habitat loss (Florida Natural Areas Inventory 2010), meaning there may have been relatively few barriers to gene flow. The dominance of the preferred habitat of *P. ionantha* and the lack of obstacles to a water dispersed species may have led to frequent gene flow across large areas, thus leading to the low levels of genetic differentiation observed today. Differences in breeding system are not likely driving the observed gap in genetic diversity between *P. ionantha* and *P. moranensis*, as both species require insect visitation for pollination (Alcalá and Domínguez 2012; Molano-Flores et al. unpubl. data), but the mating system of *P. ionantha* is uncharacterized; if *P. ionantha* experiences a large rate of geitonogamy, perhaps resulting from the inefficiency of

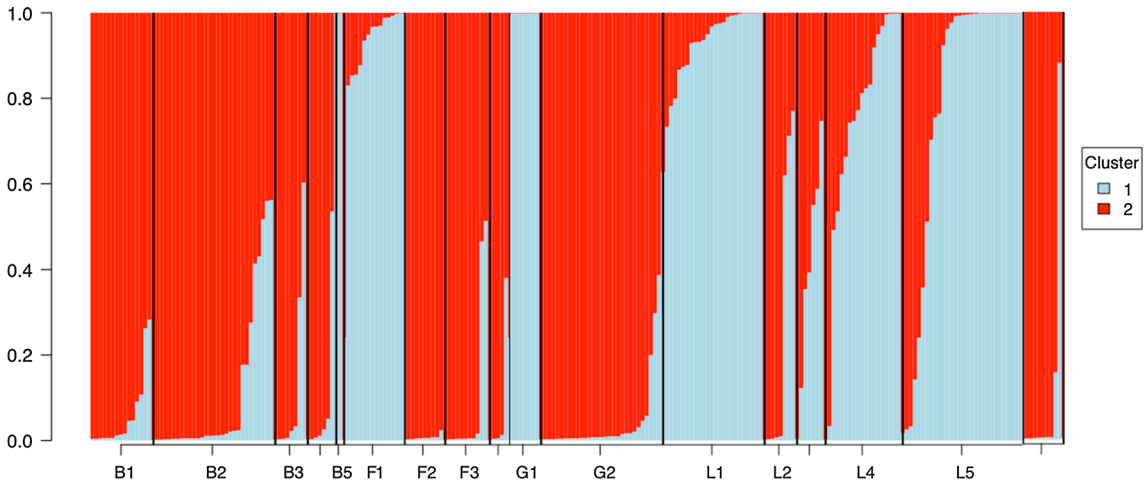

Fig. 3 Bayesian clustering results for *P. ionantha* individuals, from STRUCTURE. The model with two inferred clusters ($K = 2$) fit the data best. Each column represents a single individual, and the proportion of each individual's genome apportioned to each genetic cluster. Population names are below the figure

Table 3 The mean proportion of membership for all individuals at each study site in each of the two clusters ($K = 2$) defined by STRUCTURE

Population	Inferred cluster	
	First	Second
B1	0.941	0.059
B2	0.891	0.109
B3	0.874	0.126
B4	0.909	0.091
B5	0.001	0.999
F1	0.106	0.894
F2	0.993	0.007
F3	0.908	0.092
F4	0.919	0.081
G1	0.001	0.999
G2	0.961	0.039
L1	0.076	0.924
L2	0.734	0.266
L3	0.607	0.393
L4	0.269	0.731
L5	0.222	0.778
L6	0.893	0.107

The results shown here represent the means from three runs

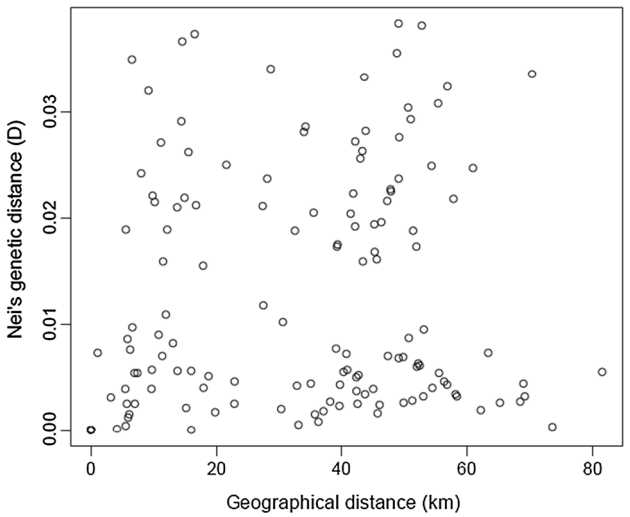

Fig. 4 Relationship between pairwise genetic and geographic distances among populations of *P. ionantha*. Mantel tests did not find significant isolation by distance ($P > 0.226$)

"buzz" pollinators (Molano-Flores, personal observation), it may lead to greater rates of selfing and decreased genetic diversity. Clonal reproduction is an unlikely driver for the low genetic diversity observed in *P. ionantha* because we did not find evidence of clonal reproduction occurring in the field (B. Molano-Flores, personal observation), nor was it mentioned in any species description that we encountered. Notably, our study uses AFLP markers while Alcalá and Domínguez (2012) use RAPDs, thus some caution should be taken when directly comparing our results. However, the review by Nybom (2004) suggests that the two dominant markers yield comparable results.

The lack of isolation by distance suggests that although *P. ionantha* populations are found within an increasingly developed and urbanized matrix over the past 70 years, gene flow is occurring or has occurred in the recent past among sites. Most sites of *P. ionantha* are found within areas with a long history of protection at the state and federal level (e.g., Apalachicola National Forest established in 1936 and Tate's Hell State Forest established in 1994), and it is not clear if the observed lack of differentiation is a relict of past connectivity (Horner et al. 2014). It is unlikely that ongoing gene flow maintains genetic connectivity between geographically distance populations, because of the highly fragmented habitat and presumed poor dispersal (V. Negron-Ortiz, USFWS; personal communication).

Monitoring of reproductive success is recommended for sites with the lowest levels of genetic diversity because deleterious effects of low diversity may suppress recruitment, and thus justify augmentation from other populations. Some of the study populations, such as those at in Liberty County, have low PLP values indicating little genetic diversity (Table 1). If there is evidence that low diversity is adversely affecting sites such as L1 and F5 augmentation with material from B5 or G1 may be the best course of action because they belong to sites that are part of the same genetic cluster (Table 3; Fig. 3). In general, the lower genetic diversity in the cluster of populations that includes B5 (Table 1; Fig. 2) may warrant additional monitoring for signs of reduced fecundity or vigor.

Of interest is the genetic clustering of populations that are geographically distant from each other. A possible explanation is that the population genetic patterns observed resulted from discrete colonization events, as opposed to ongoing gene flow. It is also possible that the genetic clusters represent genotypes adapted to different local conditions, though we did not observe differences between sites that align with the two groups and there was substantial admixture within most sites (and some individuals). Because there are no clear geographic patterns in the population genetic structure for *P. ionantha*, it may be appropriate to use criteria other than geographic proximity if establishing a new in situ population in the region covered by our study. For example, material from populations with greater genetic diversity or fewer signs of inbreeding depression may be favored over material from populations that are physically nearer. However, because there are multiple lines of evidence supporting the distinction of two genetic groups in our study area (i.e., STRUCTURE results), it may be advisable to avoid mixing genetic material between those groups due to the possibility of

outbreeding depression (Fenster and Galloway 2000; Edmands 2007) and the loss of "evolutionarily significant units" (Fraser and Bernatchez 2001).

The lack of a relationship between the vastly varying population sizes and genetic diversity is consistent with several scenarios: (1) very recent changes (probably declines, but maybe increases) in population size that have not been reflected in the perennial adults' genetics; (2) the plants are highly selfing in all habitats, thus increased population size does not greatly change the mating system; (3) the plants are obligate outcrossers, which slows inbreeding and loss of alleles due to genetic drift; (4) there is cryptic gene flow; or (5) population sizes are larger than what is being estimated by observation. Breeding system studies are underway to better understand the reproductive ecology of the species, which will allow us to assess some of these scenarios.

Considering the findings presented above, we make the following recommendations for ex situ and in situ conservation: (1) when establishing new populations of *P. ionantha* material from multiple sources (i.e., a number of different populations) should be used, in order to capture the total amount of variation observed in this species while increasing the likelihood of reproductive and demographic success (Kephart 2004); (2) it is advisable to use material from the same cluster of populations when either augmenting existing populations or establish new populations (Montlavo and Ellstrand 2001; Huford et al. 2012); (3) low levels of genetic diversity appear to be commonplace in *P. ionantha*, although special attention may be warranted for those populations with the lowest level of diversity. To conclude, this study has shown low genetic differentiation between populations, points to recent matrix connectivity even in cases of great landscape isolation among *P. ionantha* populations, and informs what strategies are most appropriate for conservation efforts.

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