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Genetic and habitat variation among populations of the critically imperiled *Vicia ocalensis* (Fabaceae) in the Ocala National Forest, USA[1]

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Abstract. *Vicia ocalensis* Godfrey & Kral (Ocala vetch) is a declining endemic plant that is state-listed as endangered in Florida and as critically imperiled globally. This perennial vine is historically known from only three populations along the shorelines of separate spring systems within the Ocala National Forest. One of these populations recently rejuvenated after being absent for 15 years. All populations have experienced great annual fluctuations in size, and are increasingly threatened by recreational use of the springs, herbicide treatment, and other habitat disturbances. The aims of this study are to assess genetic diversity within and between the three historical populations of *V. ocalensis* and characterize habitat supporting the two extant populations of *V. ocalensis* to inform conservation strategies. We analyzed 743 amplified fragment length polymorphism (AFLP) markers. Our AFLP results show evidence of high inbreeding and weak genetic differentiation among populations. Genetic diversity within Population 1 and Population 3 was lower than Population 2. Complementary monitoring data over the last 10 yr shows population size declines in these three populations. The low genetic diversity and inbreeding is a concern for this species, especially coupled with habitat loss, degradation, and fragmentation. Our analysis of habitat suggests that germination substrate is a key component in determining suitable habitat for *V. ocalensis*, but understory and overstory plants may also influence habitat suitability. Habitat protection, restoration of similar habitats, population introductions, and *ex situ* preservation are all recommended for this species.

Key words: AFLP, endangered species, endemic, population genetics, springs

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Estimates of genetic diversity provide important information to guide efforts to conserve endangered species (*e.g*., Frankham 1995, Kramer and Havens 2009, Griffith *et al*. 2013, Frankham *et al*. 2014). Maintaining diversity can protect the evolutionary potential of a species and reduce the probability of extinction (Reed and Frankham 2003, Ouborg *et al*. 2006). In small populations, inbreeding and genetic drift are risks that can reduce genetic diversity, although the effects of inbreeding and drift may be stochastic and unpredictable (Bouzat 2010). Low diversity can decrease a population's ability to adapt to changing environments (Jump *et al*. 2009, Markert *et al*. 2010). Larger populations tend to have higher fitness and genetic diversity and are therefore thought to be more resilient to changing environments (Leimu *et al*. 2006).

Vicia ocalensis Godfrey & Kral (Fabaceae) was originally described from three specimens collected at Juniper Creek, in the Ocala National Park, Marion County, Florida (Godfrey and Kral 1958), and has only been known from three populations along separate spring systems within the Ocala National Forest (Chafin 2000), although two new locations have been located during surveys in May 2018 (C.L.P., unpublished). *Vicia ocalensis* is a declining endemic that is state-listed as endangered (Ward *et al*. 2003) and globally critically imperiled (NatureServe 2015). The three historical populations are threatened by recreational use of the springs, herbicide treatment, and other habitat disturbances, and have experienced great annual fluctuations in size, with few plants observed in years where springtime water levels were unusually high or low (Peterson 2014, 2015). Annual population surveys since 1997 by Bok Tower Gardens (Lake Wales, FL) have shown that the species occurs primarily along specific locations of spring shorelines, with little annual variation in occurrence area (Peterson 2014). One of the populations has shown a gradual decline for at least 10 yr (Population 1, unpublished data C.L.P.), and plants at this site do not appear as robust as in the other remaining population. One of the populations was not observed in 2004 and remained absent until rediscovered at the site in 2018 (C.L.P., unpublished). Little else is known about this species, as there are few published studies on its reproductive biology, life cycle, habitat needs, or vulnerability to environmental threats; this information would help develop conservation strategies (Gunn 1965).

Within population footprints, habitat structure at fine scales (microhabitat) can greatly influence recruitment, survival, or reproduction of Florida's rare plant species (Maliakal-Witt *et al*. 2005, Noland *et al*. 2017), including wetland species (Vander Stelt *et al*. 2017). Regular surveys since 1997 have identified areas along the shorelines of springs where *V. ocalensis* is often present, and have distinguished adjacent areas in which it has never been observed. It is not evident through casual observations if habitat characteristics differ between these areas. The populations appear to be in decline, and vegetation along the shorelines has been affected by storms, recreation, and occasional mechanical treatment for invasive species. A better understanding of the microhabitat required for recruitment and survival of *V. ocalensis* is necessary for land managers to develop strategies to remove exotics and permit recreation without negatively impacting *V. ocalensis* populations. Microhabitat requirements can also be used to restore the habitat and to help select specific locations along additional springs for introduction efforts.

Known pollinators of the species are *Apis mellifera* L., *Bombus* Latreille spp., and *Toxomerus* Macquart sp. (Adams *et al*. 2010). The three study populations are located from 2 km to 9 km apart, and may be reproductively isolated from each other based on known foraging ranges of these pollinators. Seeds are primarily gravity dispersed, as they are ejected a short distance from the seed pod when ripe, and are thought to quickly sink or lodge into suitable substrate after dropping into the spring runs (C.L.P., personal observation). Species that have a limited ability to disperse seeds are more vulnerable to decline because of insufficient adjacent suitable habitat (Colas *et al*. 1997). Declining, isolated populations are inherently vulnerable to genetic threats of inbreeding depression and bottlenecks (Ellstrand and Elam 1993, Wright *et al*. 2013).

In situations where there are very few natural populations remaining and plant numbers continue to decline, preventing extinction often necessitates the introduction of new populations as part of the overall conservation strategy. An understanding of the genetic diversity remaining within *V. ocalensis* and between the three historically known populations, along with habitat factors that may influence

their persistence, will be key for informing conservation strategies. The aims of this study are to assess genetic diversity within and between the three historical populations of *V. ocalensis* and characterize habitat supporting the two extant populations of *V. ocalensis*.

Materials and Methods. SPECIES DESCRIPTION, STUDY SITES, AND SAMPLING PROCEDURE. *Vicia ocalensis* is a perennial vine that grows as mats and spreads horizontally and vertically by tendrils (Kral 1983). The species has lax, pale green stems, with alternate pinnately compound leaves, axillary inflorescences with 12 to 18 yellow pale flowers, and a legume of 4 cm to 4.5 cm that contains 8 to 12 seeds up to 2.5 cm wide (Kral 1983). The species is similar to *Vicia acutifolia* Elliott and *Vicia floridana* S. Watson, but has longer stems, longer leaflets, more and longer flowers, broader and longer legumes, and wider seeds (Kral 1983). *Vicia ocalensis* has a karyotype of 2n = 14 (Veerasethakul and Lassetter 1981). Morphological and karyotypic characters suggest that *V. ocalensis* is more closely related to *V. acutiflora* than to *V. floridana* (Godfrey and Kral 1958, Hermann 1960, Veerasethakul and Lassetter 1981). *Vicia ocalensis* flowers between March and May and is located in small, marshy areas in central Florida spring systems (Kral 1983). Study locations are along three spring systems within the Ocala National Forest (Fig. 1). All of these spring systems are fed by constant flow from the Floridan aquifer and feed into the same large lake. Site names and location information are omitted here to protect the populations. Population 1 is the northernmost site, Population 2 is located 3.3 km south of Population 1, and Population 3 is located 14.57 km south of Population 2. Individuals of *V. ocalensis* have not been observed in Population 3 between 2003 and 2017. We included this population in genetic analyses because it could harbor alleles lost in the extant populations, and could be a source for these alleles in restoration plantings.

DNA ISOLATIONS AND AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP). Seedlings were generated in July 2014 using seed collected between 2003 and 2011 from wild populations and stored in the seed storage facilities at Bok Tower Gardens. Seeds used in this study were sampled across these harvest years by collecting one or two pods from a branch, with each pod containing up to eight seeds. Because of the multi-branching and spreading nature of this species, individual maternal parent plants could not be distinguished during seed collection and plant count estimates within each population for each harvest year were therefore also not possible, but every attempt was made to collect from across the breadth of each population to maximize diversity. From 350 to 3,000 seeds were collected during each collection, which represented from 22 to 188 maternal parent plants. Seedlings selected for this study were sampled from across all available harvest years to further maximize diversity. Only 11 seeds, harvested in 2003, were available to represent Site 3 (site or Population 3) because too few plants remained in the population, and none was present through the time of this study; these seeds produced just seven seedlings for this study. Seeds from the absent Site 3 population were included in the study to help determine whether this population might have contributed to the remaining diversity of the species through historical gene flow, and to help protect any genetic uniqueness at this site in the event a dormant seedbank may exist to help reestablish the population following any future restoration efforts. DNA was extracted from each seedling using 100 mg of fresh leaf tissue for a total of 80 DNA samples ($n = 37$ from Site 1, 36 from Site 2, and 7 from Site 3). Leaf tissue was rinsed in distilled water, and pulverized using a plastic pestle in a 1.5-mL microcentrifuge tube filled to the 100 μL line with Garnet Matrix™ (MP Biosystems, Santa Ana, CA) and with 400 μL lysis buffer (Qiagen, Germantown, Maryland). DNA was extracted using the DNEasy Plant Mini Kit™ (Qiagen) according to the instructions of the manufacturer, and eluted in 50 μL nuclease-free water. DNA was stored at 4 °C until use.

Twelve DNA samples (representing four seedlings from each of the three populations) were subjected to selective amplification using 12 primers optimized for other *Vicia* species (Potokina *et al*. 2002, Jonsson *et al*. 2008) in order to select optimum primer pairs for this study. The AFLP procedure (Vos *et al*. 1995) was performed using AFLP Plant Mapping kit instructions (Applied Biosystems, Foster City, CA). The preamplification primers were EcoRI-A (5′-GACTGCGTACCAATTCA-3′) and MseI-C (5′-GATGAGTCCTGAGTAAC-3′) with single selective bases A and C, respectively. For the selective amplification, two additional selective

bases were added to the 3′ end of each primer in different combinations. Each of the forward primers was fluorescently labeled with FAM dye for visualization on a vertical polyacrylamide gel. The selective amplification reactions were submitted to the University of Florida, ICBR Interdisciplinary Center for Biotechnology Research Gene Expression and Genotyping Core laboratory for analysis. Four primer pairs were selected as optimum, based on total number of polymorphic peaks and clearly detectable fragments, and used for selective amplification reactions on the remaining DNA samples. The primer combinations we used were EcoRI-AGA-MseI-CAG, EcoRI-AGC-MseI-CGT, EcoRI-AGC-MseI-CGC, and EcoRI-ACA-MseI-CGT. Peaks were scored manually by three people (Y.R. and two student interns) with Peakscanner (v 1.0, Applied Biosystems).

Genetic Diversity. We used the following statistics to describe population genetics: percentage of polymorphic bands, effective number of alleles per locus, expected heterozygosity. and analysis of molecular variance (AMOVA). These metrics were estimated with GENALEX 6.3 (Peakall and Smouse 2006). To assess genetic differentiation, we used a Bayesian approach implemented in HICKORY 1.1 that has been suggested for dominant data like AFLPs (Holsinger and Lewis 2003). With this method we estimated genetic diversity within each population (hs), heterozygosity (HeB) within populations, the coefficient of inbreeding (f, analogous to F_{IS}), and population differentiation (hB, analogous to F_{ST}). Estimates were calculated under three different models: a $f = 0$ model where data are set to have no inbreeding, a $\theta = 0$ model where differentiation is forced to zero, and a full model where f and θ can vary. Five runs of each model were conducted to ensure consistency of results (burn-in 50,000, sample = 250,000, thin = 50) as suggested by Holsinger *et al*. (2002). We report the full model with f and θ because it had the best fit estimated by the deviance information criterion, a metric similar to Akaike's information criterion that takes into account the fit of a model and the number of factors used. However, as recommended in the HICKORY documentation, we use caution in interpreting f because the algorithm may not be able to accurately estimate it from dominant marker data.

Isolation by distance was assessed with the Isolation by Distance web service (Jensen *et al*. 2005). A significant correlation between log (genetic Slatkin's similarity index $M = [1/F_{ST} - 1]/4$) and log [geographic distance]) was estimated with a Mantel test based on 30,000 randomizations. Genetic association among individuals were investigated by principal components analysis (PCA) using a covariance method and was carried out in 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). Our analysis was made under the admixture model, with 1,000,000 repetitions after a burn-in of 500,000, and it was replicated 10 times. We completed this analysis for each *k* value (number of populations) ranging from 1 to 5. The most likely *k* value was estimated from the results in STRUCTURE HARVESTER (Earl and von Holdt 2012), following Evanno *et al*. (2005). Optimal cluster for the selected *k* was obtained with CLUMPAK (Kopelman *et al*. 2015).

Habitat Characterization. In August 2014, microhabitat was compared at locations supporting plants (hereafter "plant locations") to "nonplant locations" where plants had not been observed during annual surveys, based on previously used methods (Richardson *et al*. 2013, 2014). At each of the two sites supporting extant populations, microhabitat data was collected within 2-m^2 quadrats at 20 plant and 20 nonplant locations, which were selected within the boundaries of the population.

We measured the following microhabitat characteristics within each quadrat: percentage of the quadrat over water and land; maximum height of the understory vegetation; percentage of cover of potential substrate, defined by any mucky or soft organic material in which seeds could become lodged and germinate; water temperature, depth, and velocity; canopy density (spherical densiometer, Robert E. Lemmon, Forest Densiometers, Bartlesville, OK); distance to the nearest spring head; and percentage of bare sand, grasses, herbaceous plants (nongrasses), woody plants, and detritus comprising the ground cover. We determined the percentage of each type of ground cover by calculating the area of each quadrat that they covered. This area was estimated visually and quantified by assigning units based on the following system (after Richardson and Hanks 2009): 0.5 (0–1% of the total area of the quadrat),

Table 1. Genetic diversity estimates in *Vicia ocalensis* populations based on amplified fragment length polymorphism loci. n = sample size, PPL = % polymorphic loci, Na = no. of different alleles, Ne = no. of effective alleles (1/[sum π^2]), uHe = unbiased expected heterozygosity, I = Shannon's information index (–1× sum [$\pi \times \text{Ln}(\pi)$]). Means are given ± SE.

Population	n	PPL	No. of bands	No. of private bands	Na	Ne	uHe	I, Shannon's Information Index
1	37	69.58	517	58	1.39 ± 0.03	1.19 ± 0.01	0.12 ± 0.01	0.20 ± 0.01
2	36	81.29	608	128	1.63 ± 0.03	1.20 ± 0.01	0.13 ± 0.06	0.22 ± 0.01
3	7	35.80	301	49	0.76 ± 0.04	1.17 ± 0.01	0.10 ± 0.06	0.16 ± 0.01
Mean	26.67	62.23 ± 13.64	475.33	78.33	1.26 ± 0.02	1.18 ± 0.01	0.12 ± 0.01	0.19 ± 0.01

3 (1–5%), 15 (5–25%), 37.5 (25–50%), 62.5 (50–75%), 85 (75–95%), and 97.5 (95–100%). At each location we also identified the predominant species of overstory trees, midstory plants, and understory plants.

Differences between microhabitat at plant and nonplant locations were tested by separate general linear models blocked by site, including the interaction term (PROC GLM; SAS Institute 2011). We used square-root, base-10 log, and square-root arcsine transformations on data when necessary to meet assumptions of normality prior to analysis. We calculated the percentage of times five categories of plants were the most abundant ground cover within the 2-m^2 quadrat at *V. ocalensis* and nonplant locations: (a) *Cladium* P. Browne sp., (b) *Eichhornia crassipes* (Mart.) Solms, (c) other single species of plants, (d) multiple species, or (e) no plants. We calculated the percentage of times six categories of midstory plants were closest to *V. ocalensis* and nonplant locations: (a) *Cladium* sp., (b) *Myrica* L. sp., (c) *Quercus* L. spp., (d) other single species of plants, (e) multiple species, or (f) no plants. We also calculated the percentage of times seven categories of overstory trees were the nearest tree to plant and nonplant locations: (a) *Acer rubrum* L., (b) *Myrica* sp., (c) *Quercus* spp., (d) *Sabal palmetto* (Walt.) Lodd., (e) other single species of trees, (f) multiple species, or (g) no trees. Separate χ^2 contingency tests were used to determine whether the categories of ground cover, midstory, and overstory plants, calculated across the two sites, differed at *V. ocalensis* and nonplant locations.

Results. GENETIC DIVERSITY. AFLP analysis resulted in 743 fragments. The sizes of the fragments were in a range from 37 bp to 587 bp in length. Within populations, genetic variation, represented as percentage of polymorphic bands, ranged from 35.80% in Population 3 to 69.58% in Population 1 and 81.29% in Population 2. Also, private bands followed the same pattern: Population 2 had 128 whereas Populations 1 and 3 had 58 and 49, respectively (Table 1). The differences in number of different alleles between Populations 1 and 2 compared to Population 3 were nearly double, whereas unbiased expected heterozygosity, number of effective alleles, and Shannon's information index were almost the same among populations (Table 1).

Our results provide evidence of high inbreeding ($f = 0.99 \pm 0.02$) and moderate genetic differentiation among populations ($\theta[II] = 0.18 \pm 0.02$, Gst-B $= 0.13 \pm 0.01$) using the best-fitting full model in HICKORY. The interpretation of f from dominant marker data in HICKORY requires caution; however, this result is reasonable given the decline in the size and number of populations remaining of this species. Genetic diversity within each population (hs) varied from 0.19 in the smallest population to 0.18 and 0.23 in the two larger populations. Results of the AMOVA show that the molecular variance was mostly distributed within populations (87%), rather than among populations (13%) (Table 2). Genetic diversity was not correlated with distance ($R^2 = 0.9$, $P = 0.3$) (Table 3). In the PCA, the first and second components explained 8.45% and 7.11% of the total variability, respectively. Principal component analysis indicates some differentiation of Population 2. Bayesian clustering analysis with the STRUCTURE algorithm determined the best fitting K to be three, with individuals from each of the populations assigned to both of the inferred population clusters (Fig. 2).

HABITAT CHARACTERIZATION. Few of the habitat characteristics we measured differed between plant and nonplant locations. However, plant locations averaged 53% of germination substrate, whereas nonplant locations averaged 29% ($F = 14.9$, df = 1, P

Table 2. Analysis of molecular variance of 743 amplified fragment length polymorphism fragments of three populations of *Vicia ocalensis*.

Source	Degrees of freedom	Sum of squares	Proportion of variation (%)	*P*
Among populations	2	529.34	3	0.001
Within populations	77	4,502.86	87	
Total	79	5,032.20	100	

Table 3. Pairwise genetic distance based on fixation index PhiPT (above diagonal) and geographic distances in kilometers (below diagonal) between of three populations of *Vicia ocalensis* based on analysis of 743 amplified fragment length polymorphism fragments.

	Population 1	Population 2	Population 3
Population 1	—	0.11	0.19
Population 2	3.65	—	0.18
Population 3	20.65	19.16	—

< 0.001). The predominant species in the understory varied between plant and nonplant locations: *Cladium* sp. was more prevalent at plant locations, but plant locations were also more likely to have multiple species or none at all compared to nonplant locations ($\chi^2 = 41.1$, $P = 0.001$; Table 4). The predominant tree species in the overstory also varied: *Acer rubrum* and *Myrica* sp. were more prevalent and *Quercus* spp. were less prevalent at plant locations ($\chi^2 = 20.3$, $P = 0.002$; Table 4). The predominant midstory plants were not different between plant and nonplant locations ($\chi^2 = 9.0$, $P = 0.11$).

Discussion. Genetic Diversity. We accessed levels of genetic variation in three populations of *V. ocalensis* and found moderate levels of population differentiation and low genetic variation overall, consistent with a small population size across the geographically narrow range and limited gene flow between the remaining populations. No

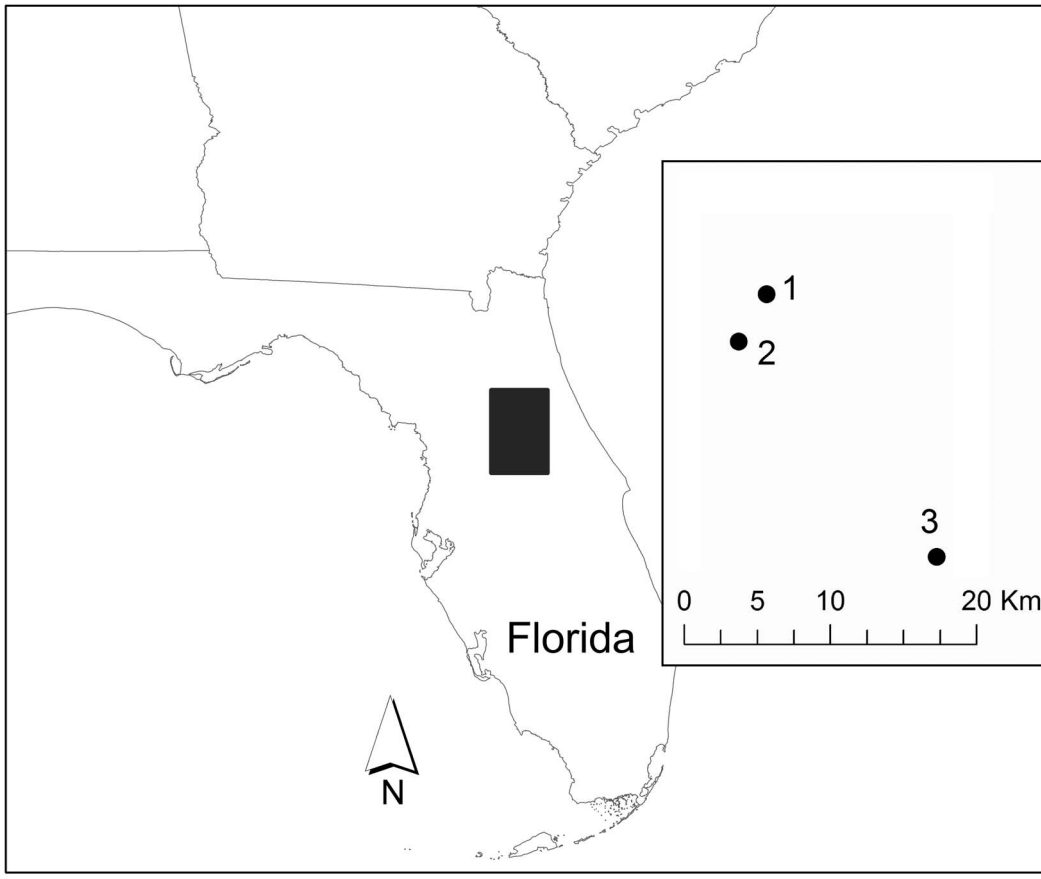

Fig. 1. Geographic distribution of the *Vicia ocalensis*.

FIG. 2. Bayesian model-based clustering analysis of three remnant populations of *Vicia ocalensis*. Each color represents different genetic group ($K = 3$) and each vertical line corresponds to each individual.

significant genetic structure was apparent, although the three populations are likely reproductively isolated. We interpret this result as indicating limited differentiation among the existing stands, consistent with dispersal occurring via waterways. The three populations are located along spring systems that feed into the same lake, suggesting they may historically have been contiguous or additional populations may have previously existed that allowed gene flow. The lack of genetic partitioning between populations and the low remaining levels of diversity suggests a strategy of mixing germplasm from the remaining populations may be optimum for enhancing fitness in future introduction efforts. Mixing seeds can increase available stocks and help retain rare alleles in restoration efforts.

Habitat fragmentation and population reduction are identified as two factors decreasing genetic diversity (Newell and Morris 2010). The genetic structure we observed in *V. ocalensis* might be associated with a reduction of population size in Population 1 (C.L.P., unpublished data) and in Population 3, which recently experienced a long-term absence of plants. The population structure does not show significant differentiation among the populations, or isolation by distance. These results are supported both by the PCA and the Bayesian clustering approach.

SUITABLE HABITAT. Our analysis of habitat suggests that germination substrate is a key component in determining suitable habitat for *V. ocalensis*. Suitable habitat also appears to be associated with certain species of understory and overstory plants. An overstory of *Acer rubrum* and *Myrica* sp. appear more favorable for *V. ocalensis* than an overstory dominated by *Quercus* spp. It seems that *Quercus* spp. may have an antagonistic relationship with other plant species because, in addition to our results in this study, it is infrequently found close to endemic understory plants in Florida, but yet well represented in nearby nonplant habitat (Richardson *et al*. 2013, 2014). Also, habitat analyses suggest the presence of *V. ocalensis* is positively correlated with several shoreline canopy species (*Myrica* sp., *Acer rubrum*) and prefers *Sabal palmetto* or *Cladium* sp. on which to climb. The latter two species can also provide the humid, wet substrate required for its seeds to lodge and germinate. A shoreline that is drier or predominated by oaks does not support this species. Habitat structure is a major factor controlling the local distribution of plants (*e.g*., Richardson *et al*. 2013, 2014). Given the extreme rarity of *V. ocalensis*, characterizing suitable habitat to inform management strategies to maintain existing populations and select locations to establish new populations is an avenue worth pursuing.

The most common threat affecting rare plants in the continental USA is outdoor recreation (Hernández-Yáñez *et al*. 2016). Each of the three studied populations exists within popular boating and swimming areas. Regular intense recreational use in the immediate population areas may impact habitat quality and change hydrology of spring runs (NatureServe 2015) and pollinator behavior, and may lead to reduced fitness and long-term viability of *V. ocalensis*. During this study, water and soil samples were collected from each far end and the center of all three populations. Preliminary analyses (C.L.P., unpublished data) have shown very high levels of lead, arsenic, and other high-population–related pollutants associated with areas in proximity to roads, parking areas, and recreational boating sites. The effects of these pollutants on the recruitment and persistence of the *V. ocalensis* and other species in this plant community have not been studied. The reason for the 15 years loss of the species at Site 3 is unknown, but if these pollutants have a deleterious effect on this species, poor habitat quality may have contributed to its decline because it is located under a heavily traveled road underpass and has high levels of vehicle-associated pollutants; the soil is more acidic at Site 3 than at the extant sites, and

Table 4. Relative frequency ± SEM that understory and tree species were the predominant vegetation nearest to locations of wild *Vicia ocalensis* and nonplant locations (*Vicia ocalensis* not present) across two sites in central Florida.

	Understory species					Tree species					
Location	*Cladium* sp. (saw grass)	*Eichhornia crassipes*	Other single species	Multiple species	No understory	*Acer rubrum*	*Myrica* sp. (wax myrtle)	*Quercus* spp.	*Sabal palmetto*	Other single species	Multiple species	No overstory
Vicia present	40.0 ± 15.0	7.5 ± 7.5	7.5 ± 2.5	25.0 ± 10.0	12.5 ± 7.5	17.5 ± 2.5	20.0 ± 0.0	2.5 ± 2.5	10.0 ± 5.0	7.5 ± 2.5	5.0 ± 5.0	37.5 ± 7.5
Nonplant	17.5 ± 2.5	30.0 ± 25.0	17.5± 17.5	12.5 ± 2.5	2.5 ± 2.5	10.0 ± 0.0	7.5 ± 2.5	10.0 ± 0.0	5.0 ± 5.0	17.5 ± 17.5	12.5 ± 12.5	32.5 ± 17.5

invasive aquatic species are abundant (C.L.P., personal observations).

Conservation Implications. Low genetic diversity and inbreeding is a concern for this species, especially coupled with habitat loss, degradation, and fragmentation. With few populations and little genetic variation within the three study populations, *V. ocalensis* is in need of active steps to preserve variation and establish new populations. According to International Union for Conservation of Nature (IUCN) criteria, *V. ocalensis* is listed as Near Threatened (Contu 2012). Despite the fact that the species is located in protected areas, because of the small geographic range and the vulnerabilities of the populations, *V. ocalensis* should be listed as Critically Endangered according to criteria B1ab(iii) (IUCN 2001).

In general, species known from a few isolated populations show low genetic diversity (*e.g.*, Oleas *et al*. 2014, Jaros *et al*. 2016) and we have observed levels of diversity consistent with this level of rarity. Our data show that understanding its mating system and maintaining the limited genetic diversity in *V. ocalensis* should be a priority for the species. Each of the three study populations differ moderately from one another and harbor distinct alleles. Actions to maintain diversity, such as maintaining *ex situ* collections, protecting and restoring habitat, and establishing new populations can help maintain existing diversity. Our data also support the need for additional efforts to maintain Population 1, which is subject to the greatest impacts from recreation and appears to be in decline.

The mating system of this species has not been explored, and whether *V. ocalensis* is self-incompatible, as is common in the Fabaceae, is not known. Although its pollinators are likely abundant, recreational use of freshwater spring systems may influence reproduction, as anthropogenic activities are thought to disturb plant-pollinator interactions, and have consequences for plant reproduction, demography, and even mating systems (Eckert *et al*. 2009).

Our findings indicate that suitable habitat for this species consists of locations along the shorelines that lack *Quercus* sp. and invasive species such as *Eichornia crassipes*; have a soft, mucky substrate to support seed germination; have a canopy of *Acer rubrum* and *Myrica* sp.; and include *Sabal palmetto* or *Cladium* sp. on which to climb. Because germination substrate was deter-

mined to be key to the presence of this species along shorelines, our recommendations are that future research efforts should focus on better defining suitable germination substrate and identifying how it is influenced by fluctuating water levels, recreational use, herbicides, and other environmental disturbances. The positively associated species may be part of a successional complex that management can encourage, although more information would be needed to support this possibility. The effect of pollutants and herbicides on seed germination, growth, and reproduction of this species needs to be understood to help land managers develop strategies for its protection. It would also be useful to have more information on the impacts of *Eichornia,* which comprised a larger percentage of the understory in nonplant locations, and can have substantial ecosystem-altering impacts. Population introductions and augmentations, especially along spring systems with limited recreational impacts, are recommended, and should be implemented to maximize preservation of the species' remaining genetic diversity at each introduction site. Coupled with the finding that there is little population structure or subdivision among the remaining populations, we believe that planting in suitable habitat can be performed with few risks of interfering with population structure.

The relationship of *V. ocalensis* to other native *Vicia* species is still poorly understood. Further work is needed to understand the potential for hybridization with other *Vicia* species, as well as the origin of *V. ocalensis*. This information will be critical to guiding potential restoration plantings, and to inferring the intensity and duration of any historical population bottlenecks.

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