

## Genetic diversity and clonality in the federally endangered plant *Clematis socialis* Kral (Ranunculaceae)<sup>1</sup>

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GOERTZEN, L. R. AND R. S. BOYD (Department of Biological Sciences, 101 Life Sciences Building, Auburn University, AL 36849). Genetic diversity and clonality in the federally endangered plant *Clematis socialis* Kral (Ranunculaceae). J. Torrey Bot. Soc. 134: 000–000. 2007—Allozyme variation in the endangered plant *Clematis socialis* and a closely related species (*C. crispa*) was examined by horizontal starch gel electrophoresis. Two populations of each species were sampled for allelic variation at seven loci to estimate genetic diversity. The relative contribution of clonal growth and seedling recruitment to maintenance of *C. socialis* populations also was estimated from the electrophoretic results by calculating genotypic diversity. Both species possessed moderate levels of genetic diversity: mean expected heterozygosity ( $H_e$ ) was 0.302 for *C. socialis* and 0.246 for *C. crispa*. Analysis of multilocus genotypic diversity revealed 26 distinct genotypes among 55 individuals of *Clematis socialis*, and 22 different genotypes for 37 individuals of *C. crispa*. The genetic diversity of *C. socialis* is more consistent with values reported for nonclonal plants, and levels of multilocus genotypic diversity suggest that considerable sexual reproduction has occurred in these populations despite low levels of observed seedling recruitment.

Key words: Allozymes, asexual reproduction, clonal growth, conservation, endangered species, genetic diversity.

Levels of genetic diversity play a significant role in determining the evolutionary potential and long-term survival of both populations and species of plants (Soltis and Soltis 1989, Godt and Hamrick 1991, Richter et al. 1994). High levels of genetic diversity are associated with the ability of populations and species to adapt to changing environmental conditions and increased resistance to diseases and pests (Barrett and Kohn 1991). Thus, empirical studies that estimate genetic diversity are important components of successful conservation and management of rare plants (Godt and Hamrick 2001).

The rich endemic flora of the southeastern U.S. contains 482 rare plant species (Estill and Cruzan 2001) yet the genetic diversity of most has not been studied. Often, priority for conservation research is given to species

threatened by extinction. For example, the state of Alabama has 12 flowering plant taxa federally listed as endangered (Alabama Natural Heritage Program 2006) and genetic diversity has been examined for some of these, e.g., *Dalea foliosa* (Gray) Barneby (Edwards et al. 2004), *Lindera melissifolia* (Walt.) Blume (Godt and Hamrick 1996), *Sarracenia rubra* Walt. subsp. *alabamensis* (F.W. & R.B. Case) Schnell (Godt and Hamrick 1998a), *Schwalbea americana* L. (Godt and Hamrick 1998b) and *Trillium reliquum* J.D. Freeman (Gonzales and Hamrick 2005). Information provided by these studies can be an important objective of Species Recovery Plans, e.g., *Schwalbea americana* (U.S. Fish and Wildlife Service, 1995).

Among the endangered Alabama plants yet to be studied genetically is the Alabama leather flower, *Clematis socialis* Kral, an extremely rare plant. Discovered in 1980 growing on a roadside in St. Clair County, Alabama (Kral 1982) it was known only from the type locality until 1985, when a second population was discovered in Cherokee County, Alabama. As of 2005, it was known from five sites in northeastern Alabama (Cherokee, Etowah, and St. Clair counties) and one in northwestern Georgia (Floyd County) (C. Norquist, pers. comm.). An additional very small population (covering < 1 m<sup>2</sup>) has been found at one additional Alabama site in Cherokee County since 2005 (R. Boyd, pers. obs.). Federally listed as endangered in 1986

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(U.S. Fish and Wildlife Service 1986), its precarious status is due in part to the proximity of all known populations to human-disturbed areas and to the relatively small area occupied by known populations (U.S. Fish and Wildlife Service 1989, Boyd and Hilton 1994). Boyd and Hilton (1994) estimated that the populations known at the time of their study occupied less than 4 ha and the additional sites discovered since their 1992 field study probably have no more than doubled this area (R. Boyd, pers. obs.).

Recent studies have begun to provide information on the biology of this endangered species. *Clematis socialis* exhibits clonal growth, an unusual feature among southeastern *Clematis* (Kral 1982). Plants sometimes form discrete patches of tightly bunched stems, but in other cases stems are more widely spaced so that it is impossible to spatially identify clones. Aerial stems emerge from belowground rhizomes in late winter (early March), flower through April, and begin to senesce in June (Timmerman-Erskine 1992). By August these aerial stems have died back to the rhizomes. Observations of marked flowering stems indicated that the next year's aerial stem is produced within about 10 cm of the stem from the previous year (R. Boyd, pers. obs.).

Investigation of pollination biology in this species (Timmerman-Erskine and Boyd 1999) demonstrated that flowers are able to set fruit (aggregates of single-seeded achenes) when insect visitors are excluded but that flowers visited by insects produced significantly more achenes. Further, hand-pollinated flowers produced still more achenes, suggesting that fruit set is pollinator limited. The most frequent floral visitors were the native bees *Bombus pennsylvanicus* (DeGeer) and *Anthophora ursina* Cresson, but the importance of these species to *Clematis socialis* pollination varied between years depending on the timing of flower production in this early-blooming plant and the flight seasons of the bee species (Wall et al. 2003). Despite observing the production of viable achenes, Timmerman-Erskine and Boyd (1999) found no evidence of population recruitment through seedlings and concluded that sexual reproduction may be limited by a combination of pollinator scarcity, herbivory, low light levels, and post-dispersal seed predation by rodents.

Although some information on the reproductive biology of *Clematis socialis* has been

gained, the relative roles of clonal and sexual reproduction in maintaining population size of this endangered species are not known. Boyd (pers. obs.) found that, under field conditions, a rhizome that produced a flowering stem in one year would usually produce only a single stem the following year, but observations of plants under greenhouse conditions suggest rhizomes can produce more aerial shoots under some conditions.

Clonal reproduction can play a significant role in the level and pattern of genetic diversity maintained in plant populations. Numerous plants reproduce both sexually and through vegetative offshoots (Richards 1997) with the relative role of sexual vs. clonal propagation varying in response to ecological and genetic factors that favor one mechanism over the other (Eckert 2002). It is of basic biological interest, and also useful for management considerations, to know the relative importance of clonal growth compared to recruitment from seedlings. The Recovery Plan for *Clematis socialis* (U.S. Fish and Wildlife Service 1989) states that the relative importance of these modes of reproduction should be determined for populations of this endangered species.

Given the lack of reported seedling recruitment, we hypothesized that *Clematis socialis* has reproduced primarily clonally and thus predicted high genetic similarity of plants within populations. To test this, we determined genetic diversity within and between the two largest known populations of *C. socialis*. By examining multilocus genotypes, we used these data as evidence of genetic recombination and thus sexual reproduction within populations. Studies of rare species often include populations of more widespread congeners in order to provide some basis of comparison (e.g., Godt and Hamrick 2001). We compared our estimates of genetic diversity for *C. socialis* with those for a widespread and relatively common species (*C. crispa* L.) that is typically placed in the same section or subgenus (Viorna) of *Clematis* (Tamura 1987). *Clematis crispa*, the swamp leather flower, ranges across the southeastern U.S., from Virginia in the north to Florida in the south and westward to Mississippi, Tennessee, and Kentucky (Radford et al. 1968). Although Kral (1982) remarked on the similarity in appearance of *C. crispa* and *C. socialis*, he noted that the rhizomatous habit of *C. socialis*

was unique. Thus, the two species differ in that *C. socialis* may be able to rely upon asexual reproduction to maintain population size.

**Materials and Methods.** **STUDY POPULATIONS.** *Clematis socialis* was sampled from the two largest populations currently known: one in St. Clair County and one in Cherokee County, Alabama. These sites are ca. 65 km apart. The St. Clair County site is located on the Nature Conservancy's Dry Creek Preserve. This population is the type locality of *C. socialis* (Kral 1982) and is located on the floodplain margin of Dry Creek. It is the largest population: Boyd and Hilton (1994) reported that 84% of all *C. socialis* plants known at that time (17,900 stems) were found at this site. Most plants in this population (92%) are located in an area of about 0.2 ha that was selectively logged in 1984, prior to the establishment of the preserve (Boyd and Hilton 1994). Other studies of the reproductive biology of *C. socialis* (Timmerman-Erskine and Boyd 1999, Wall et al. 2003) have primarily focused on this population because of its large size, protected status, and relatively natural appearance.

The Cherokee County site was the second population of *Clematis socialis* to be discovered and is located on a 400 m<sup>2</sup> section of annually mowed highway right-of-way adjacent to an intermittent creek (Boyd and Hilton 1994). Plant density is particularly high in some portions of this population, with 11% of the stems counted at all sites in 1992 (17,900 stems) occurring at the roadside portion of this site (Boyd and Hilton 1994).

The two *Clematis crispa* populations selected for sampling were located in Macon and Madison Counties, Alabama. The Macon County population is located near Hardaway and consisted of *C. crispa* plants growing in the border of woods and fields along the side of a dirt road over an approximate distance of 1 km. The Madison County population is on the grounds of the U.S. Army's Redstone Arsenal. Plants in this population were scattered in the understory of a several hectare site dominated by a deciduous hardwood overstory.

**SAMPLE COLLECTION.** Plant material was collected from the field during spring when growth was most rapid. Due to our desire to spread our sampling across individuals within

the populations, collection sites for leaf material were spaced at least 5 m apart for *Clematis socialis* populations and 8 m apart for those of *C. crispa*. Since new *C. socialis* stems are produced by a rhizome within about 10 cm from the location of a prior year's flowering stem (R. Boyd, pers. obs.), the spacing of our *C. socialis* samples prevented duplicate sampling from recent clonal growth. Leaves were placed into plastic bags and transported on ice to the laboratory. Samples were stored at 4°C until protein extraction (within 4 wk of collection in all cases).

**ISOZYME CHARACTERIZATION.** Leaf samples (ca. 1 cm<sup>2</sup>) from individual plants were ground to liquid consistency in 0.1 M Tris-HCl pH 7.5 extraction buffer (Soltis et al. 1983) or the extraction buffer of Werth (1985) modified by addition of 5 g polyvinylpyrrolidone (PVP) and 0.5 ml of 2-mercaptoethanol. Extracts were absorbed by paper wicks and loaded into horizontal 12.5% starch gels. Electrophoresis was performed at 4°C with the histidine-citrate, LiOH-borate, and NaOH-Borate continuous buffer systems of Soltis et al. (1983) and a modified LiOH-borate buffer (Ashton 1990). Gel slices were stained according to the protocols of Soltis et al. (1983) and Wendel and Weeden (1989). Interpretation of electrophoretic patterns was based on the known quaternary structure of the enzyme and its subcellular compartmentalization (Kephart 1990). For each enzyme system, the "fastest" or most anodally migrating isozyme was scored as locus "1". Within each locus, the most anodal allele was designated "a".

Allele frequencies were inferred directly from the observed electrophoretic patterns. Allozyme diversity was summarized for each species by P, percentage of polymorphic loci, A, mean number of alleles per locus, and H, average expected heterozygosity. All genetic diversity parameters were estimated from the allele frequency data by the programs BioSsys (Swofford and Selander 1981) and GeneStat (Lewis 1993).

Multilocus genotypes were examined with the program MultiLocus v.1.3 (Agapow and Burt 2001). The average number of genotypes per individual was estimated by the value G/N, where G is the number of genets (distinct multilocus genotypes) identified and N is the total number of individuals (ramets) sampled

Table 1. Values for genetic variability statistics and multilocus genotype statistics for the four *Clematis* populations sampled.

	<i>Clematis crispa</i>		<i>Clematis socialis</i>	
	Redstone	Hardaway	St. Clair	Cherokee
<i>Genetic variability statistics</i>				
Mean alleles per locus (A)	1.6	1.6	1.7	1.7
Percent polymorphic loci (P)	43%	57%	71%	71%
Observed heterozygosity ( $H_o$ )	0.107	0.320	0.085	0.338
Expected heterozygosity ( $H_e$ )	0.212	0.279	0.321	0.282
<i>Multilocus genotype statistics</i>				
Number of individuals (N)	16	21	27	28
Number of distinct multilocus genotypes (G)	10	12	14	12
Genotypes per individual (G/N)	0.625	0.571	0.519	0.429
Number of individuals with most common haplotype	4	4	6	4
Multilocus genotypic diversity	0.925	0.933	0.925	0.942

(Ellstrand and Roose 1987). Multilocus genotypic diversity was defined as the probability that two individuals taken at random would have different genotypes, using the formula:  $n/(n-1) [1 - \sum p_i^2]$ , where  $p_i$  is the frequency of the  $i$ th genotype and  $n$  is the number of individuals sampled (Agapow and Burt 2001).

**Results.** Of the 20 enzyme systems for which significant attempts at staining were made, seven were clearly resolved and provided loci that could be interpreted genetically. These were (abbreviation and staining procedure reference in parentheses): Catalase (CAT: Soltis and Soltis 1989), Glucose-6-phosphate dehydrogenase (G6PDH: Werth 1985), Glutamate dehydrogenase (GDH: Werth 1985), Isocitrate dehydrogenase (IDH: Werth 1985), Malate dehydrogenase (MDH: Werth 1985), Malic enzyme (ME: Soltis and Soltis 1989), and Shikimate dehydrogenase (SKDH: Werth 1985). Five additional loci were adequately resolved but interpretation was complicated by intractable banding patterns. Inconsistent staining or lack of staining precluded the remaining enzyme systems from this analysis.

Electrophoretic data from the seven interpretable loci, using approximately 20 individuals from each population, indicated moderate levels of genetic diversity in both species (Table 1). Mean values of  $H_e$  were 0.302 for *Clematis socialis* and 0.246 for *C. crispa*: these did not significantly differ ( $t$ -test:  $t = 1.4$ ,  $df = 2$ ,  $P = 0.29$ ). Comparisons of the number of loci with multiple alleles (P) and the average number of alleles found at each polymorphic locus (A) suggest slightly higher genetic diversity within *C. socialis* than *C. crispa*.

The observed heterozygosity for both species did not differ significantly from expected allele frequencies ( $\chi^2 = 0.15$ ,  $df = 3$ ,  $P = 0.98$ ), suggesting that the populations examined are near Hardy-Weinberg equilibrium.

To examine the extent of clonal reproduction in *Clematis socialis*, the number of unique combinations of alleles (multilocus genotypes) across all seven loci was scored. Twenty-six distinct genotypes were present among 55 individuals from populations of *Clematis socialis* (Table 1: mean G/N = 0.47), and 22 different genotypes were identified for 37 individuals of *C. crispa* (Table 1: mean G/N = 0.60). Mean G/N values did not differ significantly between species ( $t$ -test:  $t = 2.6$ ,  $df = 2$ ,  $P = 0.14$ ). In both Redstone and Hardaway populations of *C. crispa*, and the Cherokee population of *C. socialis*, as many as four individuals along a sampling transect possessed the same multilocus genotype (Table 1). In the St. Clair population of *C. socialis*, the largest population known, six of the sampled ramets shared an identical combination of alleles at each of the seven enzyme loci. While these data are consistent with these plants belonging to the same genet (i.e., being clonally produced), they could simply reflect the low resolution provided by this combination of markers. For both species, multilocus genotypic diversity, the probability that any two randomly selected individuals have different genotypes, was uniformly high ( $\geq 0.925$ ) across all populations (Table 1).

**Discussion.** Genetic variation in plant populations is strongly correlated with the ecological, demographic, and life-history character-

istics of the species (Nevo et al. 1984, Hamrick and Godt 1989). Published summaries of the plant electrophoretic literature provide a useful comparison for the results presented here. The comprehensive review of Hamrick and Godt (1989) organized genetic diversity estimates from 653 studies into 32 categories of eight ecological and reproductive traits. Hamrick and Godt's analysis revealed that geographic range and breeding system were the best indicators of species-level genetic diversity, with endemic species maintaining significantly less variation than widespread counterparts. In a review specifically targeting rare plant species, Cole (2003) summarized allozyme diversity estimates for 95 rare taxa compared to more common relatives. All species-level measures of genetic variation showed significant decreases in rare compared to common species.

In the species of *Clematis* examined here, these generalizations were not borne out. Although measures of genetic diversity did not differ significantly between these species, likely due to small sample sizes, the trend was for greater diversity in *Clematis socialis* than *C. crispa*. For example, *C. socialis* had more polymorphic loci than *C. crispa* ( $P$ , 71% vs. 50%: means of values in Table 1) as well as higher expected heterozygosity ( $H_e$ , 0.302 vs. 0.246: means of values in Table 1). In addition, *Clematis socialis* populations examined here possessed more genetic diversity than rare species on average. Cole (2003) reported mean values of  $P = 40\%$  and  $H_e = 0.142$  across the 95 studies of rare taxa reviewed.

Although our finding of substantial genetic diversity in a rare plant taxon is uncommon, it is by no means unprecedented. Gitzendanner and Soltis (2000) reported that 20% of the rare species they examined maintained equal or higher levels of genetic diversity than more common congeners. Perhaps a closer comparison can be made with the findings of Godt and Hamrick (2001), who surveyed allozyme diversity in a number of southeastern plants. Their analyses suggested that rare southeastern plants, which are on average closer to glacial refugia, had a significantly greater proportion of polymorphic loci and more alleles per polymorphic locus than endemic plant species in other areas. For example, the endangered Alabama endemic, *Sarracenia rubra* subsp. *alabamensis*, has relatively high levels of genetic diversity within its popula-

tions (Godt and Hamrick 1998a). Taken in this context, the relatively high levels of genetic diversity in *Clematis socialis* are not as surprising.

Genetic diversity in the widespread *Clematis crispa* is indistinguishable from the mean value for outcrossed plants reported by Hamrick and Godt (1989). While this suggests that no particular explanation for the trend towards lower diversity than the endemic *C. socialis* is required, several possibilities can be mentioned here. Historical events such as fluctuation in population size represent a significant source of error for predictions of genetic variation (Hamrick 1989). An event such as a population bottleneck could explain the relatively lower levels of genetic diversity of *C. crispa* compared to the endangered *C. socialis*, but would be difficult to detect and might have occurred for both species. Another possibility lies in our sampling of the two largest known populations of *C. socialis* for our study, thus potentially biasing the results towards greater genetic diversity. Further studies now underway include all known populations of this rare species to eliminate this concern. Additionally, allozyme variation represents only non-synonymous changes in the protein coding sequence that modify electrophoretic mobility and may seriously underestimate or otherwise misrepresent actual nucleotide diversity. This may have affected our results for one or both *Clematis* species.

As described above, one of the distinguishing characteristics of *Clematis socialis* is the occurrence of clonal growth, a very unusual feature among species of *Clematis* (Kral 1982). Clonality has a specific and obvious effect on the genetic makeup of populations, producing a strong association among particular alleles at presumably unlinked loci. This correlation will decline rapidly with the recruitment of new seedlings into the population (Balloux et al. 2003). In fact, relatively low levels of seedling recruitment are sufficient to maintain high levels of genotypic variation within plant populations (Watkinson and Powell 1993): Balloux et al. (2003) concluded that mixed clonal and sexual reproduction would be genetically indistinguishable from strictly sexual reproduction in many cases.

At first glance the allozyme data appear to support the hypothesis that clonality repre-

sents a significant mode of reproduction in the two large *Clematis socialis* populations examined here. The mean *C. socialis* G/N ratio is 0.47 (Table 1), indicating that over half of all genets were represented by multiple ramets. However, the distinct multilocus genotypes observed in all populations are interpreted as unique combinations of alleles that are the product of recombination among parental genotypes. Clonal diversity as estimated by multilocus genotypic diversity is relatively high in the populations sampled, suggesting that sexual recruitment is an additional regeneration mechanism for *C. socialis*. This is an interesting result given that seed set is poor and seedling recruitment has neither been observed in nature nor after experimental planting of achenes in the field (Timmerman-Erskine and Boyd 1999). It is possible that sexual recruitment was more common in the recent history of this species and that subsequent clonal growth has maintained a significant fraction of its genetic diversity. Either way we can conclude that the two populations of *C. socialis* are not made up exclusively of clones, as has been shown in numerous cases of plant species once suspected of strict clonality (e.g., Widen et al. 1994, Hangelbroek et al. 2002, Clark-Tapia et al. 2005, Jusaitis and Adams 2005). Analyses of the smaller populations of *C. socialis* will likely be worthwhile, as they might reveal different degrees of clonal vs. sexual reproduction at those sites.

A significant problem with the interpretation of the multilocus genotype analysis, however, is that we observed identical multilocus genotypes within populations of *Clematis crispa*, a species not suspected of clonal reproduction. Up to four individuals of *C. crispa* shared an identical combination of alleles at all seven loci (Table 1), suggesting that the overall variation in these enzyme systems is insufficient to distinguish genetic individuals. It is very likely that some *C. socialis* individuals that we considered to be clones (because they possessed an identical combination of alleles at all loci) could have unique genotypes upon the examination of additional loci. This would imply an even greater contribution of sexual reproduction to *C. socialis* population size than already seen here, increasing the disparity between our allozyme data and field observations concerning the lack of seedling recruitment and the

clear physical connections between ramets (Timmerman-Erskine and Boyd 1999).

This initial exploration of genetic diversity has shown an intriguingly high level of diversity in *Clematis socialis*. Our continuing study of *C. socialis* is turning to more variable, DNA-based marker systems (e.g., microsatellite loci) that should allow more precise resolution of individuals and enable us to better characterize the roles of clonal growth and sexual reproduction (e.g., Auge et al. 2001) in the population structure of this endangered species. In addition, samples from all currently known populations are being examined to provide a more comprehensive evaluation of genetic diversity. The inclusion of explicit spatial information (Brzosko et al. 2002) is critical to this ongoing analysis, as genotyping plants at regular, specified distances will reveal the spatial scale of single genetic individuals and help determine the relative roles of asexual and sexual propagation in this federally endangered species.

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