Int. J. Plant Sci. 178(1):000–000. 2017.

1058-5893/2017/17801-00XX$15.00 DOI: 10.1086/688960

GENETIC POPULATION STRUCTURE ACROSS THE RANGE OF ENDANGERED NORTHEASTERN BULRUSH, *SCIRPUS ANCISTROCHAETUS*

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Editor: Michele R. Dudash

Premise of research. Determining population structure and the spatial distribution of existing genetic variation is important for prioritizing areas for conservation of endangered species. Specifically, identifying clusters of genetically differentiated populations ensures that the genetic diversity of a species is conserved. *Scirpus ancistrochaetus*, northeastern bulrush, is a federally endangered wetland sedge, found in eight states in the northeastern United States, for which little information on genetic structure is available.

Methodology. We collected leaf samples from 96 separate wetlands spanning seven states and representing over half of all known sites ($N = 71$ sites). We sequenced eight variable loci, which were used to construct distance-based trees and calculate population-assignment probabilities to investigate population structure, and we tested isolation by distance by correlating genetic similarity with geographic distance among populations.

Pivotal results. All plants sampled from the same wetland (i.e., a population) were genetically identical with low heterozygosity, and there was little to no variation among clustered wetlands within a site. *Scirpus ancistrochaetus* was genetically structured across its range. Notably, all populations from New England were genetically identical to each other but differed from all other populations, and a similar pattern was observed for northern Pennsylvania populations. Three genetic clusters were identified, including a primarily New England cluster, a primarily Pennsylvanian cluster, and a southern Appalachian cluster, and overall, genetic distances were consistent with isolation by distance. Although genetic clusters mostly corresponded with geography, some populations did not show this geographic-genetic association, suggesting long-distance dispersal; for example, one population from West Virginia was assigned to the New England cluster. Overall, the highest genetic diversity was found within Pennsylvania and nearby states.

Conclusions. Whether similarity between geographically distant locations is due to animal-mediated gene flow or retention of ancestral alleles needs additional study. Additionally, the development of a large number of new markers may help to reveal diversity in areas such as New England with wetlands that did not have any diversity using our markers. Nevertheless, conservation of different genetic clusters at a regional scale is important for maintaining the genetic diversity of *S. ancistrochaetus*, particularly in its southern range, where the greater amount of genetic diversity suggests that this region is a reservoir of genetic variation.

Keywords: conservation, Cyperaceae, sedge, single-nucleotide polymorphism, temporary wetland, vernal pond.

Introduction

Preservation of genetic diversity is an important goal in the conservation of rare species. Populations with greater genetic diversity can respond better to environmental changes, whereas populations with low diversity are at a greater risk of extinction (Barrett and Kohn 1991; Frankham 1998, 2005). Genetic diversity is often lower in rare or fragmented species than in more abundant congeners due to intrinsic factors affecting small and isolated populations, such as genetic bottlenecking, genetic drift, and isolation (Frankham 1996; Furches et al. 2013). Moreover, the genetic diversity that exists can have a nonuniform distribution across fragmented populations as a result of low gene flow, independent genetic drift, and local adaptations, resulting in recognizable management or evolutionarily significant units (Moritz 1994). Understanding the spatial genetic structure of populations can facilitate conservation planning (Telles et al. 2003; Lee et al. 2006; Delgado et al. 2008), including the efficient selection and management of specific populations for conservation (Neel and Ellstrand 2003; Pillon et al. 2007), the incorporation of allelic uniqueness within conservation strategies (Petit et al. 1998), and the

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Manuscript received June 2016; revised manuscript received August 2016; electronically published November 16, 2016.

selection of transplants for restoration activities (Fant et al. 2008). Conserving populations that have diverged can also increase species resilience in the face of environmental change (Rice and Emery 2003). Therefore, planning for conservation activities can benefit from knowledge of both genetic diversity and genetic differentiation among populations (e.g., Petit et al. 1998; Frankham et al. 2011).

Species that are clonal can further complicate conservation efforts, as clonal growth can affect many genetic measures or characteristics (Balloux et al. 2003), including effective population size and heterozygosity (Chung et al. 2004; Meloni et al. 2013). Moreover, there can be a decrease in sexual reproduction due to increased reproductive costs with clonal growth that further decreases genetic diversity (Honnay et al. 2006; Honnay and Jacquemyn 2008). In the most extreme case, a population may entirely consist of a single genet (Jusaitis and Adams 2005; Gitzendanner et al. 2012), which demonstrates how using occurrence data as a measure of conservation success can be inappropriate in rare clonal species (Tepedino 2012). In addition, while various processes (e.g., genetic drift, bottlenecking, local adaption) can lead to strong differentiation among clonal populations (McLellan et al. 1997), these differences may not be detectable in species with long generation times (Honnay et al. 2006). Thus, caution must be taken when using census data as a marker of conservation success, as census data of population sizes can overestimate effective population size (Brede and Beebee 2006; Mandel 2010) and larger populations do not necessarily have greater diversity (Fleishman et al. 2001).

Selection of appropriate genetic measures is necessary. For example, the estimation of quantitative variation is important for understanding the ability of populations to adaptively evolve; however, neutral molecular markers do not adequately measure quantitative genetic variation (Reed and Frankham 2001, 2003). In addition, genetic diversity and fitness are only weakly related (Chapman et al. 2009). However, allelic diversity using as few as eight neutral markers can provide more information on adaptive potential than expected heterozygosity (Vilas et al. 2015).

Northeastern bulrush, *Scirpus ancistrochaetus* Schuyler (Cyperaceae), was first described by Schuyler (1962) and has been listed as endangered since 1991 by the US Fish and Wildlife Service (USFWS 1991). It has been determined to be taxonomically distinct (Schuyler 1967; ITIS 2016), and molecular studies place it in the tribe Scirpaeae (Muasya et al. 2009). *Scirpus ancistrochaetus* is diploid with $N = 27$ (Schuyler 1967), wind pollinated, and able to form sterile hybrids with *Scirpus atrovirens* (Schuyler 1964). It is distributed across ~120 sites in eight states in the northeastern United States, with the majority of populations in Pennsylvania (USFWS 2009; Cipollini and Cipollini 2011). Its principal habitat, particularly in its southern range, consists of small, temporary depressional wetlands that are embedded within a forested matrix. In its northern range in New England, its habitat more frequently includes margin wetlands along beaver impoundments. It can be found in single isolated wetlands or in one or more wetlands within a localized cluster of wetlands. In appropriate conditions, *S. ancistrochaetus* flowers regularly and sets viable seeds that are likely dispersed by animals (Carter 1993; Lentz and Johnson 1998; Cipollini and Cipollini 2011). However, population expansion within a wetland is primarily clonal from offshoots from the base of the plant and from shoots that form on the flowering culm (Bartgis 1992). Very little recruitment from seedlings has been observed (K. Cipollini, unpublished data). Generation time from clonal offshoot to seed can be as little as 1 year in optimal conditions; however, in shady environments, generation time can be much longer, as flowering is suppressed under these conditions (Cipollini and Cipollini 2011; K. Cipollini, unpublished data). Populations are separated by forested areas, sometimes by long distances, and so dispersal of pollen by wind is unlikely to result in a significant amount of gene flow, except in areas with clustered wetlands. Little is known about the mating system; the extent to which *S. ancistrochaetus* is self-fertile is unknown, although viable seeds capable of germinating have been collected from an experimentally isolated individual plant (K. Cipollini, personal observation).

Population sizes of *S. ancistrochaetus* fluctuate from year to year, as plants respond strongly to water level, which varies with rainfall in precipitation-dominated depressional wetlands (Lentz and Dunson 1998; Lentz-Cipollini and Dunson 2006). Individual plant growth and population sizes are affected by light availability that varies with the degree of canopy closure (Lentz and Cipollini 1998; Lentz and Dunson 1999; Cipollini and Cipollini 2011). Consequently, populations can experience strong genetic drift due to their small sizes and annual fluctuations (Loveless and Hamrick 1984). The species is primarily threatened by habitat loss, as small temporary wetlands are not well protected by the US Clean Water Act (Brooks and Paton 2005), but invasive species, hydrologic modification, forestry activities, canopy closure, and other disturbances also threaten populations (Cipollini and Cipollini 2011). Moreover, it is likely that climate change will impact this species through such mechanisms as hydrologic modification (Bauder 2005; Brooks 2009).

Since being listed as endangered, many additional populations, including some with many stems, have been discovered, prompting the first recommendation to reclassify *S. ancistrochaetus* as threatened (USFWS 2009). This change was suggested without any genetic information available for this species. Using a limited number of random amplified polymorphic DNA markers and DNA sequences from only 17 wetlands in Pennsylvania, Cipollini et al. (2013) found that genetic diversity within populations was low and that there was some geographic clustering of populations. However, screening of a greater number of populations across the full range of *S. ancistrochaetus* using additional genetic markers is necessary to better understand the genetic diversity and genetic structure of this species. In this study, we used DNA sequences from eight informative loci to investigate the genetic population structure of *S. ancistrochaetus* across its range to assure that management and monitoring activities incorporate genetic considerations for effective conservation of the full complement of genetic diversity.

Material and Methods

From 2010 to 2012, plants were collected from a total of 96 wetlands found across 71 sites (58% of all known extant sites) distributed throughout the range of *Scirpus ancistrochaetus* (fig. 1). Thus, in some cases a site was a single wet-

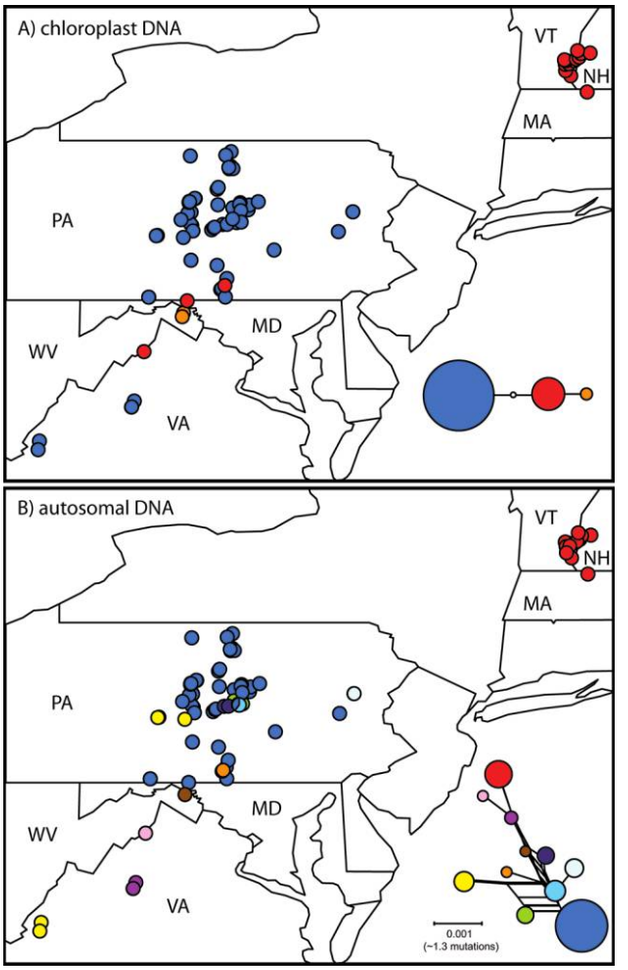

Fig. 1 Range-wide distribution of chloroplast haplotypes (*A*) and autosomal genotypes (*B*) on the basis of two and four variable loci, respectively. The haplotype network and net-neighbor diagram in the lower right-hand corner illustrates the genetic relationships among chloroplast haplotypes (*A*) and diploid genotypes (*B*), respectively. Sites with more than one genotype are indicated by multicolored circles. Symbols used in the range map correspond to the same genotype symbols illustrated in the network.

land, while in other cases a site was a cluster of wetlands found within fairly close proximity. These sites were delineated by the Natural Heritage Programs of each state. A group of plants growing in a single wetland was termed a "population" for the purposes of this study, and plants often grew in clumps within a wetland due to clonal growth. Collections were made in Pennsylvania ($N = 73$ wetlands from 50 sites), Virginia ($N = 5$ wetlands from 5 sites), West Virginia ($N = 3$ wetlands from 2 sites), Maryland ($N = 1$ wetland), Massachusetts ($N = 1$ wetland), Vermont ($N = 10$ wetlands from 9 sites), and New Hampshire ($N = 3$ wetlands from 3 sites; fig. 1). When more than one clump of plants was present, at least two samples (and usually up to 20 samples) from separate clumps were collected. While care was taken to collect plants from separate clumps, it is possible that collections were made from the same genet due to clonal growth. In some cases, such as when only one clump was present, only one sample was collected from a given wetland. Fifteen sites had between two and five clustered wetlands containing *S. ancistrochaetus*, from which samples were taken. Population sizes from the sampled wetlands ranged from five stems to over 100,000 stems, with a median size of ~300 stems. Leaf samples were placed in bags with anhydrous silica gel desiccant on collection (Fisher Scientific, Pittsburgh). We also collected samples in Vermont and New York from the related species *Scirpus expansus* Fernald (woodland bulrush; $N = 1$), *Scirpus hattorianus* Makino (mosquito bulrush; $N = 1$), and *Scirpus microcarpus* J. Presl & C. Presl (panicled bulrush; $N = 7$) to use as outgroups.

For genetic analyses, we randomly selected two individuals (generally out of 20 samples collected) per wetland when possible for a total of 180 individuals. We chose two individuals per wetland in part to screen for genetic diversity within wetlands. Although two individuals are insufficient for quantifying diversity within any one wetland, the probability of selecting identical genotypes, particularly rare genotypes, in the same wetland across all 96 wetlands is mathematically very low if clonal reproduction is unlikely (Cipollini et al. 2013). Additionally, for the 15 sites in which more than one wetland was sampled, one or two samples per wetland (depending on availability) were used for at least two samples and up to 10 samples per site. Table 1 indicates the number of wetlands and number of samples analyzed per site for sites with total sample sizes not equal to 2.

We homogenized approximately 10 mg of dried leaf material in liquid nitrogen using a mortar and pestle and extracted DNA using a DNeasy plant mini kit (Qiagen). For DNA sequencing, we used primers for four loci containing single-nucleotide polymorphisms (SNPs) identified in Cipollini et al.

Table 1

Number of Samples Analyzed in Sites When Sample Size per Site Was Not Equal to 2

Site abbreviation	No. wetlands in site	No. samples analyzed from site
AD	2	4
BBG	4	8
CHR	4	6
FLAT	4	8
GM	2	4
HARR	2	4
HPR	2	4
KEP	2	4
LM	6	10
MC	2	4
MM	4	7
MUTT	2	4
OG	1	1
RUP	1	1
SEV	2	4
ST4	1	1
STF5	1	1
ST7	3	3
TUN	2	2

(2013; table 2). We also sequenced the plastid gene rbcL, which has been used for phylogenetic studies of Cyperaceae (e.g., Muasya et al. 2009). We sequenced rbcL primarily to confirm the identity of our samples, as nonflowering individuals cannot be positively identified with certainty using field characteristics. To increase the number of nuclear markers, we screened for additional SNPs using next-generation genome sequencing data generated by Ambry Genetics (Aliso Viejo, CA). In brief, we sent two regionally pooled DNA samples (Pennsylvania and southern Appalachians), consisting of five to seven samples per pool, for 72-bp paired-end processing on an Illumina genome analyzer IIx. After quality filtering using CASAVA, version 1.7.0 (Illumina, Hayward, CA), we obtained a total of 35,000,000 reads as fastq files. A de novo assembly of reads was conducted in Geneious, version 4.8 (Biomatters, Auckland, New Zealand). SNPs were manually identified by searching assembled contigs with minor allele frequencies of ≥0.1 (this threshold was chosen to reduce the chances of identifying PCR errors as SNPs). A total of 12 SNPs, each from a different contig, were selected for further screening. Specifically, primers were designed to amplify and sequence 185–352 bp that included the targeted SNP (table 2). These 12 loci were screened for eight random samples of *S. ancistrochaetus*, and five loci were found to be useful for further study (table 2). We conducted GenBank BLAST searches to determine whether these loci matched known sequences.

Table 2

Primers Used for Screening 17 Loci in Eight Individual *Scirpus ancistrochaetus*

Locus	Primers
SA.2.08[a]	F: TCAGACTTGGTACAACCCACA
	R: TTCCAATGTTTCCCCTTTCA
SA.2.17[a]	F: CTTTGTAACGTTGTGAAGTTTGC
	R: GTCCAAGCAGGATCAGAGGT
SA.2.19[b]	F: TCTGTTGTCTTCCATCCCAAT
	R: ACAGCTCTCCACAACCCAAT
SA.2.38[a]	F: GCCTTGAATCCCTTACAACG
	R: TGCTTGCGGATAACAATCAG
SA.2.55[c]	F: GCGCTTTCCAGAGGTGTAAG
	R: TGTTTCCCTCTCGACTTTGG
SA.2.60[c]	F: TGCTGACTTTTAGGGCGACT
	R: CAAACTGCTTCACTGCGAAC
SA.2.78[c]	F: TGACGGTTCCCTACCATCAT
	R: CCAATTGCGTTTTTGATCCT
SA.2.137[c]	F: ATCACCCTCTGAGGCCTTTT
	R: GCAAGCAAGAACCCGAAATA
SA.2.168[c]	F: TTACCATGGGAGAGGCATTC
	R: AACAGGGTGGCAAGGAAAG
SA.2.337[c]	F: CAGTTAGATCCAACATCACAACC
	R: ACCTTTTTATCGGGCACTCA
SA.2.990[a]	F: TCTCAACTCCTTGCACCTCA
	R: ACATACTTTGCCCGAAGCAC
SA.4.219[a]	F: GACTGATCTTACACGGATTGAGG
	R: GAAAGCCTGTTAGAGCTCTCGT
SA.06b[a,d]	F: CTCATGCTGCTCGGCATT
	R: GAAGAAGGTTCATTGGTATTGGTT
SA.06a[a,d]	F: TTTACTTTCAGAGAAACAGGCAAC
	R: GACTTGCAGGAGCAATGGAT
SA.08[a,d]	F: CGTGTATACTCCAGCCGACA
	R: GGTAAGGAGGTGCAGAAGGA
SA.SCAM.01[a,d]	F: AGCACAAAGATCACACCTTTT
	R: AAGCACAAACTTGAGAAAACC
SA.rbcL[a]	F: GCGTTGGAGAGATCGTTTCT
	R: TCCTTTTAGTAAAAGATTGGGCCGAG

[a] Primers used in the full study.
[b] Primers yielded paralogs in *Scirpus ancistrochaetus*.
[c] Nonpolymorphic products; not assayed in the full sample.
[d] Previously published in Cipollini et al. (2013).

In total, we sequenced 10 loci, eight of which were variable, for 180 individual samples from 71 sites across the range of *S. ancistrochaetus*. We amplified DNA using methods described by Cipollini et al. (2013). Purified PCR product was cycle sequenced using ABI Prism BigDye Terminator v. 3.1 reagents (Applied Biosystems, Foster City, CA). The cycle-sequencing products were Sephadex cleaned and sequenced at the DNA Sequencing Facility at Yale University (New Haven, CT) on an ABI 3730xl DNA Genetic Analyzer (Applied Biosystems). Raw sequences were imported and edited in Sequencher, version 4.8 (Gene Codes, Ann Arbor, MI).

For each locus, we calculated nucleotide diversity (the mean number of nucleotide differences among sequences) and gene diversity (the probability of randomly sampling two sequences that are different) in DNAsp, version 5.10 (Rozas et al. 2003). Diploidy was confirmed for any locus that was heterozygous in an individual of *S. ancistrochaetus* or one of the outgroup species, and we calculated observed heterozygosity as the proportion of individuals that were heterozygous.

We concatenated all chloroplast DNA loci and all confirmed diploid loci (using International Union of Pure and Applied Chemistry [IUPAC] ambiguity codes for heterozygous positions) and constructed a neighbor-net network for each marker type separately using uncorrected *P* distances and average states for polymorphic sites in Splitstree, version 4.12.6 (Huson and Bryant 2006). We also concatenated all loci for a total of 3139 bp of sequence data, including outgroup sequences; we calculated uncorrected *P* distances (using IUPAC ambiguity codes and average states for heterozygous positions) and constructed a rooted neighbor-joining tree in Splitstree. We tested for correlations between genetic distance (*P* distances obtained from the Splitstree analysis) and geographic distance using a Mantel test in the program zt (Bonnet and Van de Peer 2002). For sites with multiple genotypes, we averaged the genetic distances among individuals. Correlations between genetic and geographic distance were tested on all populations. We then excluded the well-differentiated and geographically isolated New England populations to test for isolation by distance on a finer scale.

To estimate the number of genetic populations and assign individuals to those populations, we used two separate analyses in the program STRUCTURE 2.2.3 (Pritchard et al. 2000); one analysis included only the variable confirmed diploid loci, and the other included all variable loci. For the latter model, all chloroplast loci were concatenated and treated as a single locus to account for linkage. In addition, loci for which ploidy could not be determined were scored for a single allele, and the second allele was treated as missing data. For each locus, we coded alleles from 1 to n, where n is the number of alleles observed. To determine the number of populations (K), we estimated $\ln \Pr(X|K)$ for $K = 1$–10 populations without a

priori information regarding sampling locations. STRUCTURE was run using an admixture model and independent allele frequencies for 100,000 burn-in and 500,000 sampling generations. We replicated each analysis 10 times and calculated ΔK to determine the most likely number of populations (Evanno et al. 2005) using STRUCTURE HARVESTER (Earl and vonHoldt 2012).

On the basis of the STRUCTURE results, we defined three regions (see below). For each region, we calculated allelic richness (the number of alleles) and the number of private alleles (alleles unique to a given region). Because sample sizes differed among regions, we used rarefaction in the program HP-RARE (Kalinowski 2005) to standardize values of allelic richness and private alleles to the smallest sample size ($N = 13$ individuals). For known autosomal loci and for anonymous loci (assuming diploidy), we calculated R_{26} (expected allelic richness for $N = 26$ chromosomal copies) and P_{26} (the number of private alleles for $N = 26$ chromosomal copies), and for chloroplast DNA, we calculated R_{13} and P_{13}. We also performed a three-hierarchical analysis of molecular variance (AMOVA) to calculate the proportion of genetic variation partitioned among regions ($N = 3$), among sites within regions ($N = 71$), and among individuals within sites ($N = 180$). The AMOVA was performed in Arlequin 3.5.1.2 (Excoffier and Lischer 2010) using concatenated loci to obtain composite values of differentiation. We also calculated pairwise F_{ST} between each pair of regional groups for each locus separately in Arlequin.

Results

All samples used in our data analysis were confirmed as *Scirpus ancistrochaetus* using the rbcL gene. Among the nine other loci, only two loci matched known sequences in GenBank; SA.2.17 had 98% identity matching sequences from the chloroplast gene ATP synthase beta subunit (atpB) sampled from other Cyperaceae (e.g., GenBank accessions EU832850 and EU832851; J. M. Saarela, H. S. Rai, B. G. Briggs, A. Marchant, and S. W. Graham, unpublished data). In addition, 221 of the 290 bp of SA.2.08 had 93% identity matching a region located between the ribosomal protein L16 and L22 genes sequenced from the *Carex siderosticta* chloroplast (GenBank accession KP751906; J. Jung, J. Park, and S. Kim, unpublished data). The remaining seven loci were anonymous. Five loci were heterozygous in either *S. ancistrochaetus* or one of the outgroup species, indicating diploidy (tables 3, 4). In contrast, the remaining two loci (SA.4.219 and SA.08) contained polymorphisms across samples, but none of our sequenced individuals were heterozygous.

We found between one and five alleles per locus, with an average number of 2.5 ± 1.27 alleles (SD; table 3). Nucleotide diversity ranged between 0.0 and 0.0081 substitutions/site among loci, averaging 0.0017 substitutions/site ± 0.0024 (SD). Average nucleotide diversity in *S. ancistrochaetus* was much lower than in *Scirpus microcarpus* (mean = 0.0059 substitutions/site ± 0.0064 [SD]; table 4). Four of the five known diploid loci deviated significantly from Hardy-Weinberg equilibrium (table 3). When two individuals were sampled from the same wetland, they always had identical genotypes. Likewise, out of the 15 sites with more than one adjacent wetland containing *S. ancistrochaetus*, only three sites (MC with two sampled wetlands, MM with four sampled wetlands, and LM with five sampled wetlands) contained different genotypes within a site.

Concatenating the three chloroplast loci (1644 bp in total; rbcL, SA.2.08, and SA.2.17) resulted in three polymorphic sites defining three haplotypes (fig. 1*A*). The most common haplotype was found in 145 (80.6%) of the individuals and was widespread throughout Pennsylvania and Virginia (fig. 1*B*). The second most common haplotype was found in 31 (17.2%) of the individuals. All 26 individuals sampled from the New England states of Vermont, New Hampshire, and Massachusetts harbored this haplotype. However, it was also found in three sites within the southern part of the range of *S. ancistrochaetus*, with one site each in Pennsylvania, Maryland, and West Virginia (fig. 1*A*). Finally, the third haplotype was rare, being found in only four individuals sampled from two neighboring sites in West Virginia (fig. 1*A*). Overall nucleotide diversity for chloroplast DNA was 0.00048 substitutions/site.

Concatenating the five confirmed autosomal loci (1301 bp; SC.2.38, SC.2.990, SA.01, SA.06a, and SA.06b) resulted in eight polymorphic sites defining 11 different genotypes. Similar to chloroplast DNA, the most common concatenated geno-

Table 3

Genetic Diversity, Ploidy, Number of Alleles, Observed Heterozygosity (H_{obs}), Expected Heterozygosity (H_{exp}) Assuming Diploidy, and Nucleotide Diversity (Nucl. Div.) of 10 Loci Sequenced for 180 Individual *Scirpus ancistrochaetus*

Locus	Length (bp)	No. alleles	H_{obs}	H_{exp}	Nucl. div.	Ploidy	Location
SA.2.08	290	3	na	na	.0012	Haploid	Chloroplast
SA.2.17	296	2	na	na	.0011	Haploid	Chloroplast atpB gene
SA.2.38	294	2	.000[a]	.190	.0006	Diploid	…
SA.2.990	352	5	.089[a]	.515	.0034	Diploid	…
SA.4.219	185	2	.000	.144	.0008	Unknown	…
SA.06b	276	2	.000[a]	.144	.0005	Diploid	…
SA.06a	251	3	.000[a]	.312	.0013	Diploid	…
SA.08	236	4	.000	.582	.0081	Unknown	…
SA.SCAM.01	128	1	.000	.000	.0000	Diploid	…
SA.rbcL	822	1	na	na	.0000	Haploid	Chloroplast rbcL gene

Note. na = not applicable; unknown = a lack of heterozygosity in both *Scirpus ancistrochaetus* and the outgroup taxa.

[a] Significant deviation from Hardy-Weinberg equilibrium ($P \leq 0.001$).

Table 4

Genetic Diversity, Ploidy, Number of Alleles, Observed Heterozygosity (H_{obs}), Gene Diversity (H) Assuming Diploidy, and Nucleotide Diversity (Nucl. Div.) of 10 Loci Sequenced for Seven Individual *Scirpus microcarpus*

Locus	Length (bp)	No. alleles	H_{obs}	H	Nucl. div.	Ploidy	Location
SA.2.08	290	1	na	na	.0000	Haploid	Chloroplast
SA.2.17	296	1	na	na	.0000	Haploid	Chloroplast atpB gene
SA.2.38	294	2	.429	.363	.0012	Diploid	…
SA.2.990	352	5	.167	.167	.0115	Diploid	…
SA.4.219	185	1	.000	.000	.0000	Unknown	…
SA.06b	276	4	.714	.747	.0095	Diploid	…
SA.06a	251	2	.143	.143	.0074	Diploid	…
SA.08	236	4	.000	.810	.0121	Unknown	…
SA.SCAM.01	128	4	.857	.714	.0169	Diploid	…
SA.rpcL	822	1	na	na	.0000	Haploid	Chloroplast rbcL gene

Note. na = not applicable; unknown = a lack of heterozygosity in both *Scirpus ancistrochaetus* and the outgroup taxa (the data were consistent with haploidy).

type sequenced from 97 (53.9%) of the individuals was widespread throughout Pennsylvania and Maryland, and the second most common genotype was sequenced from 26 (14.4%) of the individuals and was restricted to and fixed within the New England states of Vermont, New Hampshire, and Massachusetts (fig. 1*B*). The third most common genotype was found in 14 (7.8%) of the individuals and was recovered from four sites in central Pennsylvania and southern Virginia. These four sites also shared identical sequences at the anonymous loci SC.08 and SC.4.219 that were not found elsewhere among our samples. The remaining eight genotypes were either unique to one site or found in neighboring sites throughout central Pennsylvania and southward (fig. 1*B*). Overall nucleotide diversity at the five known autosomal loci was 0.00141 substitutions per site.

Analyzing the four polymorphic autosomal loci in STRUCTURE, both Ln (Pr|K) and ΔK peaked at $K = 3$ populations. Under this model, all except nine individuals were assigned to one of the three clusters, with >0.98 posterior probability (fig. 2*A*). A three-population model was also supported when including chloroplast DNA and the two loci for which haploidy could not be determined (seven polymorphic loci in total). In general, the strength of assignments increased with these additional loci and the signatures of admixture decreased (fig. 2*B*, 2*C*). All individuals from New England and one site from West Virginia were assigned with ≥0.99 posterior probability to a single cluster (fig. 2*B*, 2*C*). Forty-six sites from Pennsylvania (including the three sites containing two different genotypes) and one from Maryland were assigned to a second cluster with ≥0.95 posterior probability, and an additional two sites from Pennsylvania and one from West Virginia were assigned to this cluster with >0.80 probability (fig. 2*B*, 2*C*). Widely separated sites from southern Virginia and west-central Pennsylvania were assigned to a third cluster with >0.99 probability (fig. 2*B*, 2*C*). Finally, two sites from central Virginia and one from eastern Pennsylvania appeared to be admixed with >0.30 assignment probability to both the New England and the Pennsylvania cluster, and two sites from central Pennsylvania appeared to be admixed between the Pennsylvania and the southern Appalachian cluster (fig. 2*B*, 2*C*).

Based on the STRUCTURE results and geographic proximity, we defined three regions: New England (including Massachusetts, Vermont, and New Hampshire), Pennsylvania (including all sites from Pennsylvania, Maryland, and northeastern West Virginia), and the southern Appalachians (including Virginia and the more western site from West Virginia). Note that support for a genetic southern Appalachian region population was weak (sampling sites were assigned to all three genetic clusters), but they are grouped here because sample sizes are small. Similarly, the two sites from western Pennsylvania that were assigned to the southern Appalachian cluster were grouped with the other Pennsylvania samples based on geographic proximity.

Within New England, we did not detect any genetic diversity ($R_{26(13)} = 1.0$), and only a single allele at one locus was unique to this region ($P_{26} = 0.34$; locus SA.06a). Genetic diversity was higher within both Pennsylvania and the southern Appalachians (mean $R_{26(13)} = 2.25$ and 2.14, respectively). Pennsylvania harbored slightly more private alleles than the southern Appalachians (mean $P_{26(13)} = 0.44$ and 0.25, respectively). Overall, 68.4% of the total genetic variation was partitioned among these three regions, whereas 37.8% and 3.7% were partitioned among sites within regions and among individuals within sites, respectively. Furthermore, F_{ST} was significant between each pair of regions for most loci (table 5).

Concatenating all 10 loci, we found a total of 15 different sequences (fig. 3). The most common concatenated sequence was recovered from 39 populations throughout Pennsylvania but not in any other state (fig. 3). Among the 13 populations from New England, we did not find genetic diversity at any locus, and the concatenated sequence was unique to this region (fig. 3). Although this sequence clustered with one site in West Virginia (PRU, differing at two nucleotide positions in locus SA.06a), it was distantly related to sequences at all other populations. In addition, two concatenated sequences from Virginia were identical to sequences found near the western boundary of the range of *S. ancistrochaetus* in Pennsylvania (defining the southern Appalachian cluster found in STRUCTURE analyses).

The geographic association of genotypes resulted in a significant correlation between genetic and geographic distance for all populations ($r = 0.757$, $P < 0.0001$, $n = 71$ sites). Excluding the well-differentiated New England populations did

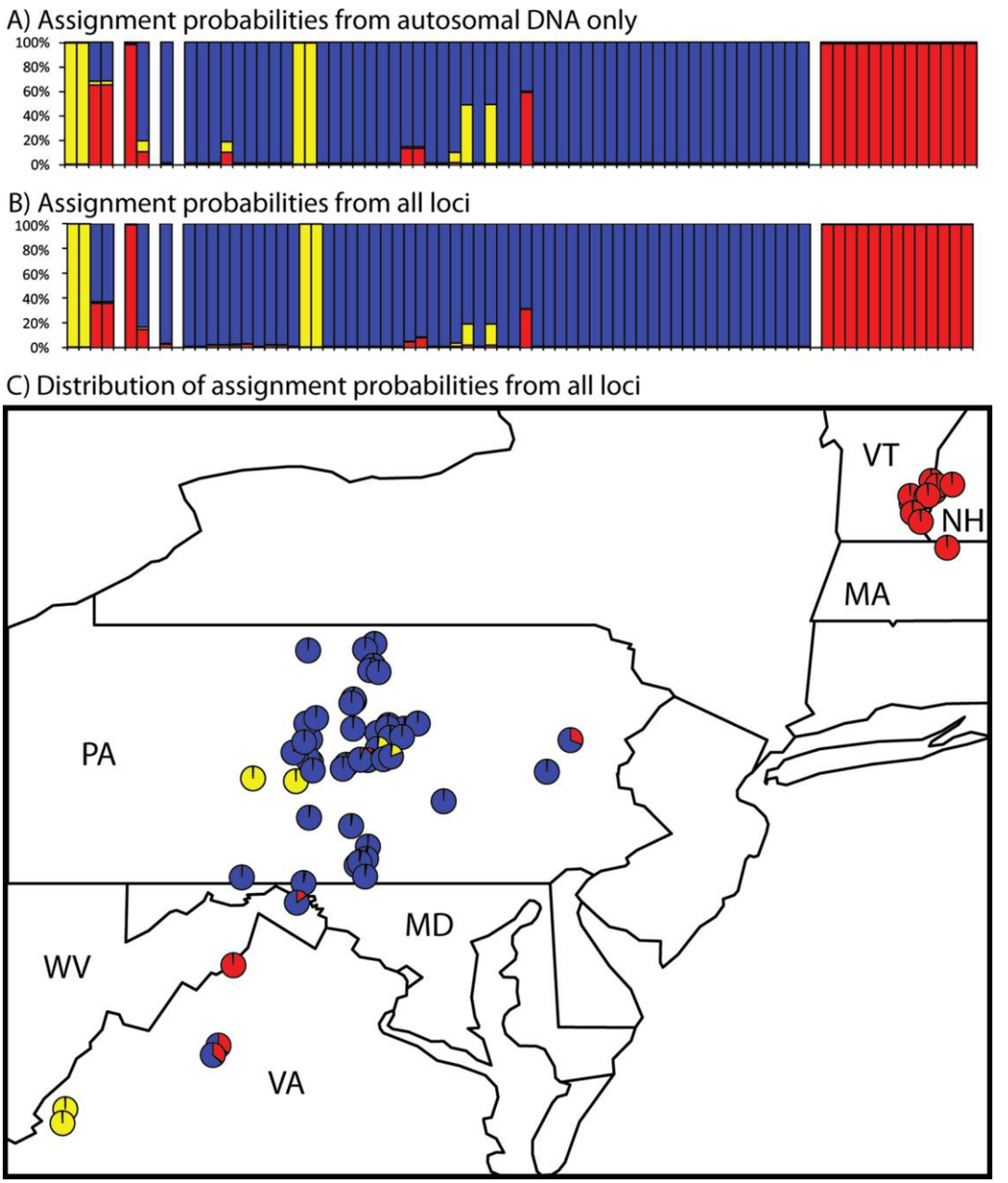

Fig. 2 Population assignment probabilities for 71 sites determined from four variable diploid loci (*A*) and all seven variable loci (*B*) under the best-supported model of population structure ($K = 3$ genetic clusters). The distribution of assignment probabilities are shown for the seven-locus model (*C*). Multiple individuals were included when a site contained more than one haplotype/genotype (one site for the diploid-only model; three sites for the seven-locus model). The two variable chloroplast loci were concatenated and treated as a single locus in the latter model (*B*, *C*).

not dramatically change this result ($r = 0.578$, $P < 0.0001$, $n = 58$ sites).

Discussion

Scirpus ancistrochaetus is listed as federally endangered, yet there is very little information about the distribution of genetic diversity and population structure. Such information is needed to guide conservation efforts. We found that *S. ancistrochaetus* is genetically structured across its range, suggesting distinct population units that are not well connected by gene flow (cf. Loveless and Hamrick 1984) and consistent with a pattern of isolation by distance. Furthermore, we identified three genetic clusters, and with a few exceptions, these clusters corresponded to geography (New England, Pennsylvania, and the southern Appalachians). The vast majority of

Table 5

Estimates of Pairwise F_{ST} between Regions of *Scirpus ancistrochaetus* Defined Based on STRUCTURE Analyses

	NE vs. PA	NE vs. SA	PA vs. SA
Chloroplast:			
SC.2.08	.866	.878	.030
SC.2.17	.907	.878	.054
Composite	.886	.878	.040
Autosomal:			
SC.2.38	.054	.418	.185
SC.2.990	.845	.418	.717
SC.06a	.919	.917	.204
SC.06b	.031	.418	.298
Composite	.808	.679	.602
Unknown ploidy:			
SC.4.219	.020	.400	.285
SC.08	.526	.844	.575
Overall	.505	.815	.558

Note. See figure 2 for details. Values are given for each polymorphic locus and the composite values for chloroplast and autosomal loci. Underlined values are statistically significant at $P < 0.05$. NE = New England; PA = Pennsylvania; SA = southern Appalachians.

genetic diversity was partitioned among these three regions, with considerably less diversity being partitioned among sites within clusters and almost no diversity found within sites. Indeed, all individuals sampled from the same wetland and most individuals sampled from the same site were genetically identical. Populations from New England and across northern Pennsylvania were each homogeneous. The homogeneous group of genotypes found across northern Pennsylvania may represent the leading edge of a northward range expansion, which can result in a loss of allelic diversity through repeated founder effects (Perisod and Besnard 2007). In contrast, southern Pennsylvania harbored more genetically diverse populations that were in close proximity. Despite the homogeneity of northern Pennsylvania, the Pennsylvania region overall had higher allelic richness and more private alleles than the other geographic regions, suggesting that it may be a reservoir of diversity for the species.

Isolation by distance is found in species with low dispersal ability coupled with habitat specificity (Louy et al. 2007). While seeds of *S. ancistrochaetus* may disperse long distances attached to animals (such as birds, bears, and deer), successful recruitment by seeds appears to be extremely low (K. Cipollini, unpublished data). Similarly, gene flow between wetlands via pollen is likely low in a forested matrix relative to open habitats and is often further reduced due to forest fragmentation (Knapp et al. 2001; Culley et al. 2002). We cannot determine whether population structure is due to adaptive divergence via habitat specificity (Jusaitis and Adams 2005) or due to genetic drift. However, the observation that populations in New England are found in habitats that differ from those in its southern range (Cipollini and Cipollini 2011) suggests that local adaption may be important. There are no studies examining local adaptation in this species except for Lentz and Dunson (1998), who reported no ecotypic variation among five populations in Pennsylvania in their growth responses to variation in water level. In addition to a more comprehensive phenotypic study across the range of *S. ancistrochaetus*, a more complete genomic assessment to identify genes likely under environmental selection will be required to assess whether any populations have become locally adapted (Savolainen et al. 2013).

Despite the presence of geographic structure, there was some genetic evidence consistent with long-distance gene flow. In particular, two populations from western Pennsylvania shared identical genotypes with populations in southern Virginia, and these four sites were assigned to the same genetic cluster, with high posterior probability. Similarly, one site in West Virginia was more similar to populations in New England than to neighboring populations in West Virginia, Virginia, and Pennsylvania; this population was also assigned to the New England genetic cluster to the exclusion of other neighboring sites, with high posterior probability. Finally, sites in northern Virginia and eastern Pennsylvania contained evidence of genetic admixture between the New England cluster and a Pennsylvanian cluster (more limited evidence of admixture was observed in other sites as well). Avian dispersal, particularly by waterfowl, and mammalian dispersal of seeds is possible given that the barbed seeds readily adhere to clothing (K. Cipollini, personal observation). However, it is also possible that alleles defining those genotypes were once more widespread throughout an ancestral population and that different contemporary allele frequencies resulted from independent genetic drift in isolated populations (Funk and Omland 2003). If this were the case, individuals from disparate locations might share alleles by random chance, although the probability of sharing alleles at numerous loci seems low under this scenario.

As in Cipollini et al. (2013), we detected no variation within any one wetland with any two randomly sampled individuals. Moreover, at the 15 sites containing multiple wetlands clustered near each other, some with as many total samples as 10, only three sites from Pennsylvania showed variation among wetlands. It is important to note that our results may be constrained in part by the low number of samples used per wetland. We chose to sample more sites across the range of the species, rather than sampling fewer wetlands more intensively. However, the probability that the same genotype, particularly a rare genotype, being randomly sampled twice per wetland is mathematically very low (Cipollini et al. 2013). Low within-population variation can be a result of many factors, including weak dispersal and strong genetic drift (Loveless and Hamrick 1984). The fluctuating nature of population sizes of *S. ancistrochaetus* (Lentz-Cipollini and Dunson 2006) should favor genetic drift in populations (Loveless and Hamrick 1984). A high rate of selfing (Hamrick and Godt 1996; Mable and Mandel 2007) or clonal growth (Jusaitis and Adams 2005; Gitzendanner et al. 2012) can also lead to low within-population variation. Experimentally isolated individuals can produce viable seed capable of germinating (K. Cipollini, personal observation), although it is unknown whether seeds are produced by self-fertilization or through apomixis. Clonal growth is the primary mode of reproduction for this species (Bartgis 1992) and is a common strategy in wetland plants of low-nutrient environments (van Groenendael et al. 1996). Our data are consistent with a colonization event by a single genotype, the initial seedling recruitment strategy of clonal plants (Eriksson 1989), followed by high amounts of asexual reproduction and restricted

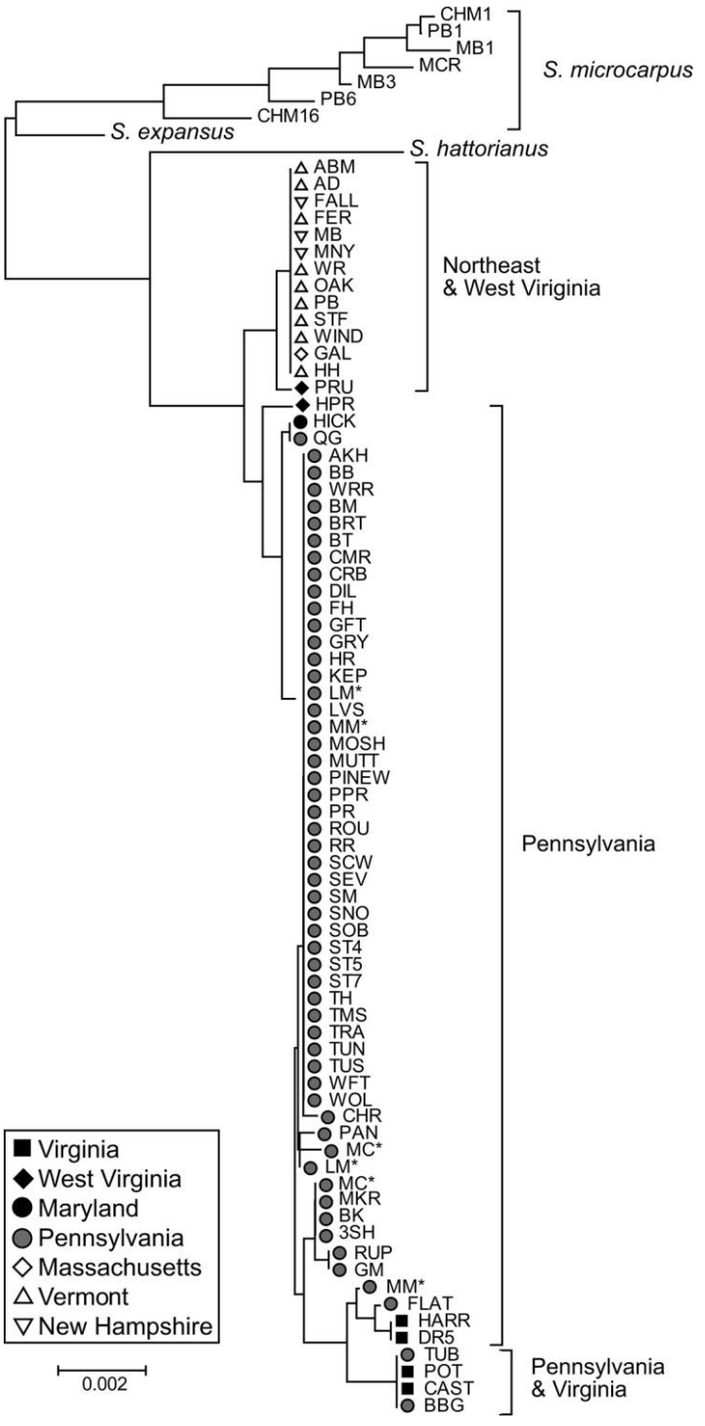

Fig. 3 Neighbor-joining tree of concatenated genotypes at eight variable loci. Asterisks highlight sites with multiple wetlands for which more than one genotype was found. Brackets group samples from the same genetic cluster as identified in STRUCTURE (fig. 2). In any single wetland, only one genotype was found. A color version of figure 3 is available online.

gene flow between wetlands. This contrasts with greater diversity seen in populations of clonal species founded by many genotypes (Goertzen et al. 2011). Furthermore, as expected for rare species (Chung et al. 2010), *S. ancistrochaetus* had less sequence variation for the markers used compared to other related species, despite a limited sampling effort. Clearly, more research needs to be done in a comparative context, but nevertheless, our results provide a glimpse at relatively low genetic diversity in this rare species.

It is important to note that our results might be constrained by the low number of markers used. Determination of genetic

structure at a finer scale can be achieved with the development of more markers. However, our results illustrate broad patterns of regionalization of common alleles, which is informative from a conservation perspective. As the only existing genetic information on this rarely studied species, our data provide important information that can be used for conservation planning and management purposes until further data are available. Information on genetic differentiation between populations, amount of gene flow, and amount of inbreeding can inform conservation decisions (Ottewell et al. 2016). Current conservation activities primarily include protection, with some movement toward habitat management through reduction of tree canopy cover; there are currently no breeding or reintroduction activities for this species. We recommend that at least two regional groups that largely correspond to identifiable genetic clusters (i.e., New England and Pennsylvania/southern Appalachians) be considered as important evolutionarily significant units (Moritz 1994) to ensure that the genetic diversity of this endangered plant is conserved. Although populations in New England harbor less variation, the unique genotypes found there are in habitats that differ from those in its southern range (Cipollini and Cipollini 2011), suggesting the possibility of adaptive divergence. Given that we found identical (or nearly identical) genotypes between the different regions, it seems likely that gene flow was relatively recent, but timing of gene flow is difficult to measure (Strasburg and Rieseberg 2011). There are differences in environmental conditions between New England and southern populations (Cipollini and Cipollini 2011), yet the differences may not be large enough to warrant management of these populations separately due to the risk of outbreeding depression (Frankham et al. 2011). Using the Frankham et al. (2011) decision tree for predicting outbreeding depression combined with the best current knowledge of this species, it is likely that outbreeding depression is not a concern and that the restoration of gene flow may provide a genetic rescue effect (Tallmon et al. 2004). However, there are uncertainties in determining the probability of outbreeding depression for this species, such as the degree of differences between habitats, the timing of gene flow, and the generation time of separation. The use of composite provencing during any reintroduction activities may be a viable conservation strategy until a better assessment of outbreeding depression, including test crosses, can be performed (Broadhurst et al. 2008).

Additionally, attention should be given to protecting the allelic diversity found in the southern Appalachian Mountains of Pennsylvania through Virginia. There are several examples of a genotype represented by a population from only one to three wetlands, such as the population in Maryland, and high priority should be given to protection and management of these populations. Conservation management may include activities such as canopy thinning (Cipollini and Cipollini 2011) or designation as a conservation area. Additional attention should be given to populations in West Virginia and Virginia due to the isolation of populations in this area. Although we sampled all known extant populations of *S. ancistrochaetus* in these states, surveys for additional populations are needed to better assess the degree of isolation. This region contains nearly as much allelic richness and as many private alleles as the Pennsylvania region, and the loss of these small, isolated populations could lead to a loss of genetic differentiation and existing genetic diversity (Butcher et al. 2009). Future genetic work should look toward using genomics to identify conservation units and provide greater detail on any within-population variation (Funk et al. 2012).

Acknowledgments

We thank the Pennsylvania Wild Resource Conservation Fund (PAWRCF), the Western Pennsylvania Conservancy (WPC), the Pennsylvania Department of Conservation and Natural Resources/Pennsylvania Natural Heritage Program (PADCNR/PNHP), and the US Fish and Wildlife Service (USFWS) for their roles in funding this work. Greg Czarnecki and Teresa Witmer of the PAWRCF, Carole Copeyon and Pamela Shellenberger of the USFWS, Greg Podniesinski of the PADCNR/PNHP, Jeff Wagner of the WPC, and Chris Firestone of the PADCNR/Bureau of Forestry (BOF) provided cheerful and prompt assistance during grant development, administration, and implementation. Ephraim Zimmerman of the WPC; Susan Klugman of the PADCNR/PNHP; Scott Bills, Rob Criswell, Bert Einodshofer, Art Hamley, Mike Ondik, and Bruce Metz of the PGC; Amy Griffith, Steven Hoover, Bob Merrill, and Jim Smith of the PADCNR/BOF; Fred Huber of the USFS; Kieran O'Malley of the WVDNR; Chris Frye and Donnie Rohrback of the MDNR; Cathy Milholen, Johnny Townsend, and Bryan Wender of the VADCR; Sara Cairns of the NHDRED; Bryan Connolly of the MADFW; Bob Popp of the VTFWD; and Bob Anderson provided valuable assistance in finding or accessing field sites. Jennifer Garrett, Mal Gilbert, Bob Popp, and Gregg Stewart were invaluable in providing plant samples and field site information. We thank Craig Chapman of the PADCNR/BOF, the Nature Conservancy, the USFS, the VADCR, the NHDRED, Barbara Douglas and Barry Parrish of the USFWS, Brandi Moyer and Richard Palmer of the PGC, and numerous land owners for permitting research or plant collection on various properties. We are also indebted to our field assistants Emmett Cipollini and Otto Cipollini.

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