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Genetic diversity among accessions of the endangered box huckleberry (*Gaylussacia brachycera*) based on AFLP markers

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POOLER, M. R., R. L. DIX (USDA/ARS U.S. National Arboretum, Floral and Nursery Plants Research Unit, 3501 New York Ave., NE, Washington, DC 20002), AND R. J. GRIESBACH (USDA/ARS U.S. National Arboretum, Floral and Nursery Plants Research Unit, 10300 Baltimore Ave., Beltsville, MD 20705). Genetic diversity among accessions of the endangered box huckleberry (*Gaylussacia brachycera*) based on AFLP markers. J. Torrey Bot. Soc. 133: 439–448. 2006.—The box huckleberry (*Gaylussacia brachycera*) is a slow-growing, dwarf evergreen groundcover that is native to eight states in the Eastern United States. It is a rare plant with conservation status of rare to critically imperiled. Genetic relationships among 24 accessions of *G. brachycera* were determined using 66 polymorphic AFLP markers from 8 primer pairs. Accessions collected in western Virginia were the most distantly related to the other accessions, while accessions from Kentucky were the most variable. The information gained from this study will be useful to guide decisions regarding conservation, preservation, breeding, and re-introduction of this species.

Key words: AFLP, amplified fragment length polymorphism, conservation genetics, endangered plant, *Gaylussacia brachycera*, genetic variation.

The box huckleberry (*Gaylussacia brachycera* (Michx.) Gray) is a slow-growing, dwarf evergreen woody groundcover that is native to both the uplands and coastal plains of Delaware, Pennsylvania, Maryland, Virginia, West Virginia, Kentucky, Tennessee, (USDA NRCS 2006) and was recently found in North Carolina (Wilber and Bloodworth 2004). A member of the family Ericaceae, *G. brachycera* may represent an isolated lineage in the *Vaccinium* and *Gaylussacia* clade, based on phenotypic and molecular characteristics (Camp 1941, Floyd 2002). The box huckleberry is usually found growing in dry or well-drained acidic soils or duff in partial shade. It has glossy, dark green, fine-textured foliage with deep red to maroon new growth and may have value as a commercial nursery plant. The box huckleberry's global conservation status is listed as G3 (rare); the state listing for Delaware, Maryland and Pennsylvania is S1 (critically imperiled), and that for Virginia and West Virginia is S2 (imperiled) (Center for Plant Conservation 2006). In Maryland, there is only one very small clump of plants left of the known wild population. In Delaware, only three wild populations have been found. In 1971, 32 different colonies were sampled from sites in the eastern United States (Smith and Smith 1971); there are an estimated 100 sites thought to contain this species (Crable 1999).

The reproductive biology of box huckleberry is poorly understood. Based on previous reports, our own observations, and breeding behavior in other related genera, *G. brachycera* is likely self-incompatible (Coville 1919, Dirr 1998, Wherry 1922). Thus, for an isolated plant where outcrossing is not possible, the only method of reproduction is by means of runners. It has been suggested that most of the populations are colonies of a single clone; however, there is no scientific data to support this suggestion other than its self-incompatibility. It is possible that genetic variation has been introduced into isolated colonies by means of chance mutations or, more unlikely, hybridizations with related species (e.g., *Vaccinium*; Coville 1919). The largest known colonies of *G. brachycera* are located in Pennsylvania, and based on the large colony size and an assumption of growth by runners only, it has been hypothesized that this clone could have existed for 13,000 years (Krussmann 1977, Willaman 1965), thereby making it the longest-lived individual on earth.

Our lab began studies of *G. brachycera* in 1998 when a Memorandum of Understanding between the Maryland Department of Natural

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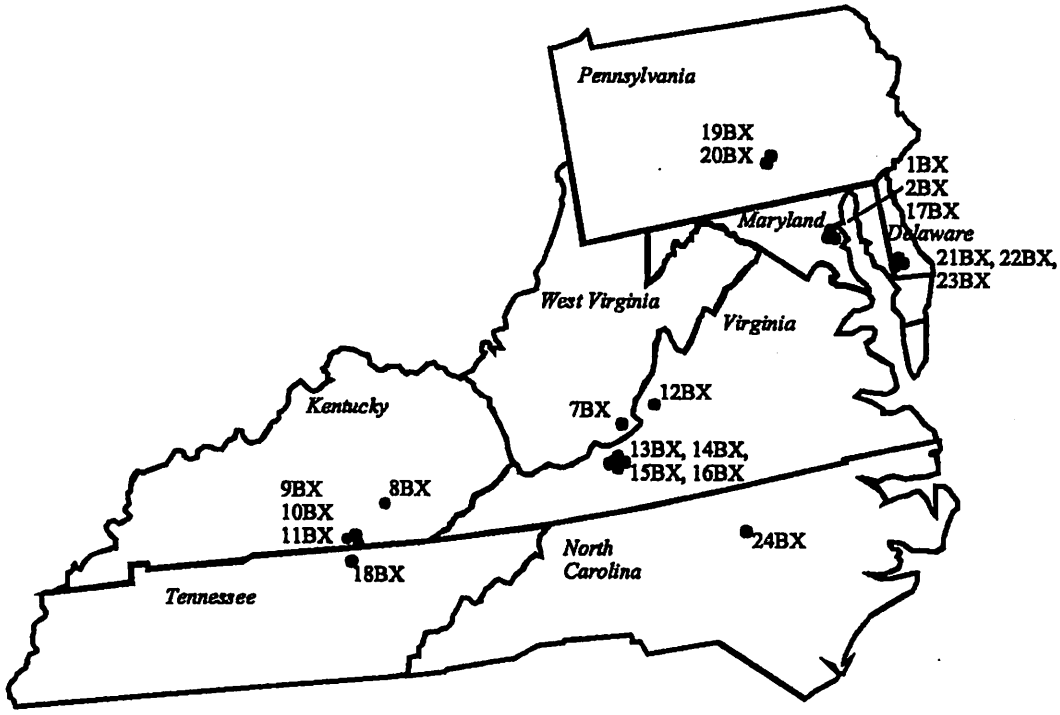

FIG. 1. Map of collection sites for those *G. brachycera* accessions evaluated in this study whose collection site is documented. See Table 1 for further collection information.

Resources and the U.S. National Arboretum was established to propagate and therefore safeguard the last remaining plants in Maryland. Since then, plants have been collected under permit from 14 native habitats in seven states (Fig. 1), and are being grown at the National Arboretum in Washington, D.C. for evaluation, breeding, conservation, and reintroduction. This collection represents one of the most extensive collections of living *G. brachycera* plants collected from over all of its known native range. Although phenotypic differences among these accessions are apparent, it is not clear how much diversity exists among these clones, nor how closely related they are. The purpose of this study was to use molecular markers (AFLPs) to estimate the genetic diversity of this collection to guide decisions regarding conservation, preservation, and breeding of this intriguing rare native plant.

Materials and Methods. PLANT MATERIALS. The accessions listed in Table 1 (except 24BX, which we obtained as silica-dried leaf material) were grown at the U.S. National Arboretum in Washington, D.C., either in the greenhouse in Anderson flats containing perlite and milled sphagnum (2:1, v/v), or in beds in a lath house containing Fafard Nursery Mix (Agawam, MA) amended with compost (9:1) and mulched with shredded leaves. Leaves for DNA analysis were taken from newer growth when possible, and were freeze dried and stored in a −80 °C freezer until analysis.

DNA EXTRACTION. DNA was extracted from three freeze-dried leaves of each accession using the methods described for other species in our laboratory (Pooler et al. 2002), with the following modification: Leaves were first ground to a powder in the lysing matrix (Bio101, Vista, CA) by vortexing, and 500 µl of prewarmed (65 °C) grinding buffer (Wilson et al. 1992) was added. The mixture was processed in a FastPrep machine (Bio101), centrifuged for 1 minute in a microcentrifuge, and the grinding buffer was pipetted off. Five hundred µl of CTAB buffer and 1.5 µl betamercaptoethanol were then added to the pelleted ground plant material, and our standard DNA extraction procedure was

Table 1. Accession information for the 24 *G. brachycera* samples used in this study.

Accession number	Collection information/origin	Date collected
NA 70976 1 BX	Lake Waterford Park, Anne Arundel Co., MD	October 29, 1998
NA 70977 2 BX	Lake Waterford Park, Anne Arundel Co., MD (dead)	October 29, 1998
NA 70945 3 BX	From National Clonal Germplasm Repository ID = CGAY7, from Tennessee	Fall, 1993
NA 70946 4 BX	From National Clonal Germplasm Repository ID = CGAY8, from Tennessee	Fall, 1993
NA 71555 5 BX	Purchased from Atlantic Star Nursery, Forest Hill, MD; thought to be from New Bloomfield, PA.	Purchased March 2001
NA 71556 6 BX	Purchased from Roslyn Nursery, Dix Hills, NY	Purchased April 2001
NA 71192 7 BX	Hollywood Township, Monroe Co., WV.	August 26, 2001
NA 71195 8 BX	Laurel River, Dorthae, KY 300 m elevation	October 3, 2001
NA 71196 9 BX	The Gulf, Daniel Boone National Forest, KY 305 m elevation	October 3, 2001
NA 71198 10 BX	Yahoo Falls Scenic Area, Daniel Boone National Forest, KY 390 m elevation	October 3, 2001
NA 71199 11 BX	Grassy Gap Ridge, Funston Benchmark, KY 342 m elevation	October 4, 2001
NA 72091 12 BX	Cowpasture River, VA, North face of ridge, 1254 m elevation	March 11, 2002
NA 72092 13 BX	Little Walker Mtn, VA. North face of fire road along toe slope #1, 2620 m elevation	March 12, 2002
NA 72093 14 BX	Little Walker Mtn, VA, in the fire road along toe slope #1, 2604 m elevation	March 12, 2002
NA 72094H 15 BX	Little Walker Mtn., VA, North face of toe slope #2, 2510 m elevation	March 12, 2002
NA 72095 16 BX	Little Walker Mtn., VA, North face of toe slope #3, 2534 m elevation	March 12, 2002
NA 72114 17 BX	From Arnold Arboretum AA1128-68; Magothy Creek, Pasadena, Anne Arundel Co., MD	Received November 2001
NA 72115 18 BX	From Arnold Arboretum AA651-69; East Jamestown, Fentress County, TN	November, 1997
NA 72116 19 BX	From Arnold Arboretum AA568-95; New Bloomfield, Perry County, PA. Collected as seed	October, 1995
NA 72117 20 BX	From Arnold Arboretum AA841-88; New Bloomfield, Perry County, PA	August, 1988
NA 72891H 21 BX	Nanticoke State Wildlife Area, Bethel, DE	November 21, 2002
NA 72891J 22 BX	Nanticoke State Wildlife Area, Bethel, DE	November 21, 2002
NA 72892 23 BX	White River Estates, Bethel, DE	November 21, 2002
24 BX	Northern Durham County, NC (leaf samples only)	December, 2003

followed. It was found that processing the leaves in this grinding buffer prior to CTAB extraction was necessary for DNA recovery.

AFLP Reactions. Amplified Fragment Length Polymorphism (AFLP) electropherograms were generated for accessions using protocols described by Vos et al. (1995) and Perkin-Elmer Applied Biosystems (1996), with slight modifications as described below, for analysis on an ABI 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA). DNA restriction and ligation were carried out sequentially, instead of simultaneously, using approximately 0.25 µg of genomic DNA. Restriction digestions were performed at 37 °C for two hours and ligations were performed at 20 °C for two hours. Preselective reactions took place in 20 µl volumes containing PCR buffer (Barry et al. 1991), plus 3 mM $MgCl_2$, 100 µM dNTP, 0.125 µM each preselective primer (One Trick Pony Oligos, Ransom Hill Bioscience, Ramona, CA), 0.625 U of Taq DNA polymerase (Promega Corp., Madison, WI), and 4 µl diluted restriction/ligation reaction. Completed preselective reactions were diluted with 380 µl molecular grade water and 3 µl were used as template for all selective reactions. Selective amplification reactions were carried out in 20 µl volumes containing the same reagents as for preselective amplification except that 0.25 µM *Mse*I primer and 0.1 µM *Eco*RI primers (Table 2) were used instead of preselective primers. The *Eco*RI

Table 2. List of AFLP selective primer extensions used and number of polymorphic markers per primer pair detected for 24 *G. brachycera* accessions.

EcoRI selective primer extension with dye name	MseI selective primer extension	Number of polymorphic markers detected
ACC (FAM)	CAC	14
ACC (FAM)	CTC	12
ACG (NED)	CAC	9
AAC (NED)	CTC	5
AGC (HEX)	CAA	9
AGC (HEX)	CAC	3
AGC (HEX)	CTG	8
AGG (HEX)	CTG	6

selective primers had fluorescently labeled 5′ ends and were purchased from the Applied Biosystems Custom Oligonucleotide Synthesis Service (Foster City, CA). Completed selective reactions were analyzed on an ABI310 with POP4 polymer. The sample was prepared by mixing 1.5 µl of selective reaction, 0.07 µl ROX size standard, and 10.15 µl deionized formamide. Markers were scored with Genotyper® 2.5 software (Applied Biosystems). All reactions were repeated from the DNA extraction through the selective reactions to ensure reproducibility of results.

DATA ANALYSIS. Similarity coefficients between each accession were calculated using the SIMQUAL program in NTSYS-pc, version 1.70 (Rohlf 1992), using the Dice similarity coefficient [2a/(2a+b+c), where a = total number of bands shared by both individuals, b = bands unique to one individual, and c = bands unique to the other individual]. These data were subjected to cluster analysis using the UPGMA method in the SAHN program of NTSYS to generate a phenogram. Cophenetic matrices were constructed and compared with the similarity matrices using the MXCOMP program to test the goodness of fit of a cluster (Rohlf 1992). Bootstrap analysis using 5000 replications was performed on the original raw data using WinBoot (Yap and Nelson 1996) to determine confidence limits of clusters in the UPGMA-based dendrograms (Felsenstein 1985). The bootstrap value indicates the percentage of times the group to the right of the node occurred in the bootstrap analysis.

Results and Discussion. AFLP MARKER ANALYSIS. Analysis of eight AFLP primer pairs revealed 66 polymorphic characters (average 8.25 polymorphic bands per primer pair), with a range of three to 14 polymorphic bands per primer (Table 2). Reproducibility between replicate samples was good, with approximately 5% of markers not used due to inconsistencies between samples. Monomorphic bands (typically over 100 per primer pair) were not scored, since they are not informative. The level of polymorphism detected in our study was low compared to studies in some species. For example, AFLP studies revealed an average of over 60 polymorphic bands per primer in a single oak species (Dodd and Kashani 2003), over 20 polymorphic bands per primer in grapevine (Fanizza et al. 2003), and 17 polymorphic bands from one primer in a self-compatible, facultative apomictic *Rhododendron* (Escaravage et al. 1998). However, in other species like *G. brachycera* that are either endangered or endemic only to a limited geographic area, our results were typical. Studies in the family Araucariaceae from Australia (Peakall et al. 2003), the triploid perennial *Limonium cavanellesii* from Spain (Palacios and Gonzalez-Candelas 1999), and the woody ornamental *Lagerstroemia fauriei* from Japan (Pooler 2003) all reveal levels of polymorphism comparable to or lower than the values found in this study.

Pairwise comparisons of accessions using these 66 markers were computed using the DICE coefficient. As seen in other studies (Koopman et al. 2001, Mace et al. 1999a, Mace et al. 1999b), the DICE coefficient combined with UPGMA clustering resulted in identical phenograms as the Jaccard coefficient, with similar correlation coefficients (data not shown). These pairwise comparisons indicated relative genetic similarities between accessions ranging from 0.51 to 0.99 (Table 3). These similarity values were then used to construct a phenogram using UPGMA clustering. Although phylogenetic analysis of closely related species using AFLPs has proven successful with other plant genera (e.g., Beardsley et al. 2003, Després et al. 2003, Hodkinson et al. 2000, Kardolus et al. 1998, Koopman et al. 2001), a phenetic approach was used in this study because our primary objective was to determine the genetic relationships among accessions within a single species, rather than to determine phylogenetic histories. In addition, it has been demonstrat-

Table 3. Genetic similarities among 24 *G. brachycera* accessions based on the Dice similarity coefficient.

1BX	1.00																							
2BX	0.99	1.00																						
3BX	0.71	0.72	1.00																					
4BX	0.81	0.82	0.86	1.00																				
5BX	0.73	0.74	0.63	0.74	1.00																			
6BX	0.70	0.71	0.69	0.76	0.71	1.00																		
7BX	0.74	0.75	0.67	0.81	0.69	0.72	1.00																	
8BX	0.78	0.76	0.71	0.78	0.68	0.70	0.80	1.00																
9BX	0.71	0.72	0.72	0.79	0.74	0.82	0.73	0.79	1.00															
10BX	0.75	0.76	0.71	0.78	0.70	0.76	0.74	0.81	0.82	1.00														
11BX	0.68	0.69	0.69	0.74	0.68	0.71	0.70	0.73	0.77	0.70	1.00													
12BX	0.59	0.60	0.60	0.63	0.57	0.51	0.61	0.49	0.55	0.56	0.57	1.00												
13BX	0.71	0.72	0.62	0.72	0.69	0.66	0.79	0.65	0.64	0.68	0.69	0.63	1.00											
14BX	0.66	0.67	0.57	0.67	0.64	0.61	0.77	0.62	0.59	0.62	0.63	0.60	0.95	1.00										
15BX	0.73	0.74	0.61	0.74	0.68	0.71	0.81	0.67	0.66	0.70	0.68	0.62	0.95	0.90	1.00									
16BX	0.69	0.70	0.60	0.72	0.70	0.67	0.83	0.66	0.65	0.72	0.67	0.61	0.90	0.92	0.92	1.00								
17BX	0.89	0.90	0.79	0.89	0.73	0.81	0.86	0.83	0.79	0.83	0.70	0.62	0.77	0.72	0.79	0.78	1.00							
18BX	0.74	0.75	0.76	0.83	0.72	0.69	0.74	0.71	0.76	0.74	0.72	0.61	0.67	0.61	0.66	0.65	0.80	1.00						
19BX	0.72	0.73	0.73	0.78	0.68	0.70	0.77	0.69	0.68	0.75	0.70	0.66	0.76	0.72	0.78	0.77	0.77	0.68	1.00					
20BX	0.73	0.74	0.63	0.78	0.94	0.76	0.75	0.70	0.77	0.70	0.71	0.58	0.71	0.67	0.73	0.72	0.78	0.75	0.68	1.00				
21BX	0.83	0.85	0.71	0.78	0.73	0.70	0.71	0.78	0.71	0.72	0.70	0.59	0.68	0.62	0.70	0.66	0.86	0.71	0.75	0.75	1.00			
22BX	0.78	0.79	0.68	0.79	0.70	0.70	0.72	0.75	0.71	0.70	0.71	0.55	0.68	0.62	0.70	0.66	0.84	0.72	0.72	0.76	0.93	1.00		
23BX	0.89	0.90	0.76	0.89	0.75	0.72	0.79	0.77	0.73	0.74	0.70	0.68	0.73	0.68	0.75	0.71	0.91	0.79	0.77	0.77	0.89	0.87	1.00	
24BX	0.83	0.85	0.74	0.81	0.73	0.76	0.83	0.81	0.76	0.81	0.70	0.59	0.80	0.75	0.82	0.81	0.92	0.74	0.80	0.75	0.81	0.78	0.83	1.00
	1BX	**2BX**	**3BX**	**4BX**	**5BX**	**6BX**	**7BX**	**8BX**	**9BX**	**10BX**	**11BX**	**12BX**	**13BX**	**14BX**	**15BX**	**16BX**	**17BX**	**18BX**	**19BX**	**20BX**	**21BX**	**22BX**	**23BX**	**24BX**

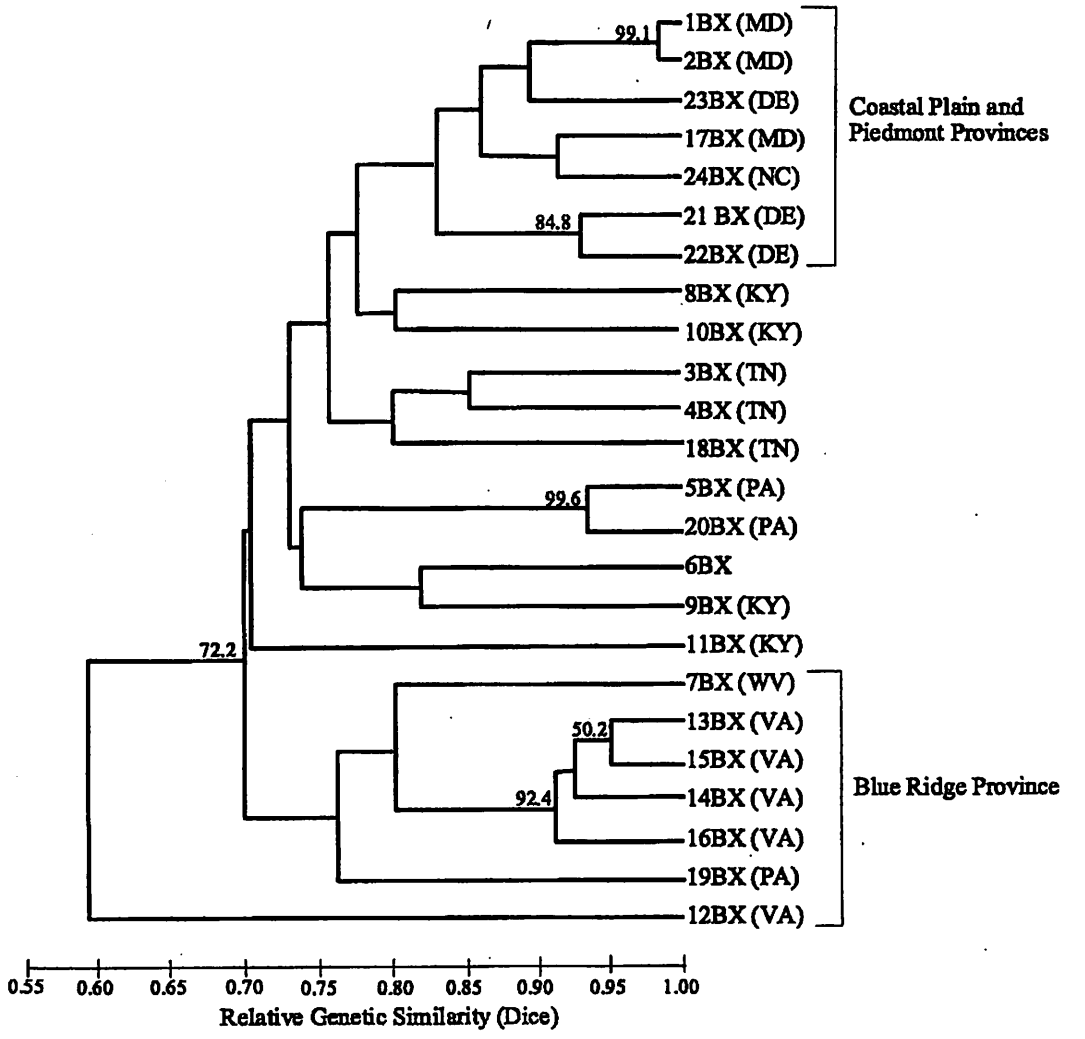

FIG. 2. UPGMA-derived phenogram of genetic similarity based on the Dice similarity coefficient among *G. brachycera* accessions based on 66 characters from 8 AFLP primer pairs. Cophenetic correlation coefficient $r = 0.834$. Bootstrap confidence values greater than 50% are indicated to the left of each node and represent the percentage of times that cluster appeared in the consensus tree.

ed that branches or clusters in a cladogram or phenogram that are well-supported by bootstrap statistics will be similar regardless of the method used (Kardolus et al. 1998, Koopman et al. 2001). The UPGMA-based phenogram using the DICE similarity data had a cophenetic correlation coefficient (r) of 0.834 (Fig. 2), indicating a fairly good fit of the phenogram with the original distance matrix (Rohlf 1992). Phenograms based on the single-link or complete-link clustering method were similar to the UPGMA dendrogram, but had lower correlation coefficients of 0.748 and 0.670, respectively. Despite a fairly high correlation coefficient, the bootstrap values were low (< 50%) for many of the branches of the consensus tree, indicating that some of the groupings may be an artifact of the clustering technique rather than a reflection of the true genetic relationships among accessions (Yap and Nelson 1996). In other words, the clustering represented in Fig. 2 describes only one of several possible phenograms. Thus, to determine genetic distances between individual clones, it may be most accurate to make pairwise comparisons of similarity values (Table 2) rather than to rely on clusters that are not well supported. The low bootstrap

values could be caused in part by the relatively low number of characters used in the data set, which has been shown to affect the accuracy of genetic distance data (Fanizza et al. 2003). However, despite the low bootstrap values of the dendrogram, many of the accessions clustered according to geographic origin.

GENETIC RELATIONSHIPS AMONG ACCESSIONS. Based on this data, the group of *G. brachycera* accessions that is the most distantly related to the other accessions consists of the plants collected from the George Washington National Forest in Virginia, 12BX–16BX, along with the accession from West Virginia (7BX) and an accession reported to have been collected as seed in Pennsylvania (19BX). Accession 12BX is not only clearly distinct from the other accessions collected from the same general geographic area (13BX–16BX), but it also forms an isolated cluster from all the other accessions analyzed. Although accession 15BX is recorded as a single accession, four plants were collected and analyzed from this large clump. The AFLP markers used in this study were not able to distinguish differences among these plants. Future research efforts in our lab will focus on sampling more plants from this and other areas to determine whether all plants from a single clump are indeed clonal, as presumed, or whether they represent distinct genotypes. However, due to the relatively low level of AFLP polymorphism present in the species, many AFLP primer pairs would likely need to be screened in order to detect differences.

Another interesting group of accessions is formed by the Maryland plants, and, in a larger context, the Maryland and Delaware plants. Accessions 1BX and 2BX were the sole surviving plants in Maryland, identified by the Maryland DNR for conservation. They were located in close proximity on a dry sandy hillside and were therefore presumed to be clonal. However, our DNA analysis has given consistent evidence that these two plants may have been distinct genotypes. Unfortunately, the smaller plant (2BX) died several years ago, and we were not successful in propagating it. Therefore, it is not possible to replicate the AFLP analysis with another DNA extraction, so we cannot say unequivocally that these two accessions were not clonal. Records from the Arnold Arboretum, where we obtained 17BX, indicate that this accession may have been collected from a site located near the surviving Maryland 1BX accession. Our DNA analysis supports this relationship (Table 3). The accessions collected in Delaware (21BX, 22BX, and 23BX) appear to be closely related to the Maryland accessions also, based on their clustering in the phenogram (Fig. 2) and the relatively high similarity coefficients between accessions (Table 3). Thus, although there is only one surviving clone in the wild in Maryland, it appears that ample related germplasm has been preserved from this area. The accession from North Carolina (24BX) is interesting in that it represents the first report of *G. brachycera* in North Carolina (Wilber and Bloodworth 2004). Although geographically separate from the Maryland and Delaware plants, the AFLP profile of this accession placed it solidly in the Maryland-Delaware cluster. Without further field work, it is not clear whether this clone is part of a larger population, an isolated colony, or even an escape from cultivation or relatively recent introduction from more northern populations.

Plants of *G. brachycera* from the New Bloomfield, PA area are probably the most well-known of the species, as it is this colony that is cited as possibly the oldest living thing on earth (Krussmann 1977, Willamen 1965). The New Bloomfield accession that we obtained from the Arnold Arboretum (20BX) was collected from the site in 1988. Another accession, 5BX, is sold commercially and is also thought to have been collected originally from this site. Our DNA analysis supports this assertion. Although the DNA profiles of these two accessions (5BX and 20BX) are similar, there were distinct differences between these two clones. Thus, if these two accessions are truly from the same site in New Bloomfield, PA, it would appear that distinct genotypes are present at this site. Although accession 19BX was also collected from Pennsylvania, it does not appear to be closely related to the New Bloomfield accessions. One plausible explanation for this distance could be the fact that accession 19BX was collected as seed and would therefore be expected to be distinct from the parental material. The fact that 19BX was collected as seed provides evidence that more than one clone is present in the Pennsylvania colony, as the species is reported to be self-incompatible (Coville 1919, Wherry 1922). Studies are currently underway in our

lab to determine the level of diversty within this colony.

The plants collected in Kentucky (8BX–11BX) are interesting in that they do not form a distinct group, despite their relative geographic proximity (Fig. 2). This diversity may be a result of sexual reproduction and geographic isolation, thus indicating that plants from these clumps may have a greater propensity for reproduction by seed. Again, molecular analysis using samples from many plants within a single site may help to determine whether these large clumps of plants are clonal.

GENETIC DIVERSITY AND CONSERVATION NEEDS. Today, *Gaylussacia brachycera* is found in four of the five Mid-Atlantic physiographic provinces (Appalachian Plateau, Valley and Ridge, Blue Ridge, Piedmont and Coastal Plain). Each of these provinces has a different soil type and environment due to the way in which it formed. The DE and MD ecotypes are found in the Coastal Plain Province. The NC ecotype is found in the Piedmont Province. The PA, VA, and WV ecotypes are found in the Blue Ridge Province. The KY and TN ecotypes are found in the Appalachian Plateau Province. Based upon this biogeography, it is possible that additional wild populations may be found in the NJ and VA Coastal Plain Province.

A number of factors could have contributed to the relatively low level of genetic diversity seen among our accessions of *G. brachycera*. Isolated populations that reproduce primarily by vegetative means often have declining genetic diversity over time, leading to fixation of a single genotype in the absence of mutation or migration (Maynard Smith 1971). This effect could be more pronounced in small populations such as are seen in *G. brachycera* where genetic drift would play a more prominent role (Lacy 1987). Additionally, in small populations it has been shown both in theory and in practice that the population size and fitness will decline over time and can eventually lead to extinction (Rottenberg and Parker 2003, Stephens and Sutherland 1999).

Numerous studies have addressed the effects that loss of diversity has on a species and on an ecological community. Recent studies indicate that the loss of even rare plants from a community can render the community more susceptible to invasion by exotic species (Lyons and Schwartz 2001). Thus, conservation of *G. brachycera* may have a broad ecological impact. In order to preserve the remaining diversity in endemic *G. brachycera* populations, it is important that these populations be protected. On-going studies in our lab using multiple accessions collected from a single site suggest that the colonies may not be composed of a single clone; therefore, it is important to conserve several plants within a colony. If several populations are lost, the resulting bottleneck effect would likely be difficult to overcome considering the propensity of the species for vegetative versus sexual reproduction. Preservation of these accessions in a protected germplasm repository and creating new clones by controlled hybridizations among accessions is the first step in ensuring that this diversity is not lost.

In order to maintain wild populations, it is important to have as much genetic diversity as possible without contaminating the gene pool. Our data suggest that for the purpose of reintroduction to create new stands in a Coastal Plain habitat, one could release a seedling population composed of MD, NC and VA ecotypes. For reintroduction into a Blue Ridge or Appalachian Plateau habit, a population composed of VA, TN, KY, and WV ecotypes could be used.

Molecular markers can be of tremendous value in *G. brachycera* ex situ germplasm conservation. They can be used to maximize genetic diversity in controlled hybridizations and breeding and to identify duplicate or near duplicate genotypes in the collection. The information on genetic relationships revealed by molecular markers can be used to drive decisions regarding re-establishing plants in the wild, and can also be used to monitor the genetic diversity in endemic and newly established populations. Furthermore, the markers can be used to answer basic questions about the reproductive biology and genetic structure of the populations of this rare native plant.

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