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RESEARCH ARTICLE

Genetic diversity of *Magnolia ashei* characterized by SSR markers

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Abstract
The Ashe magnolia *(Magnolia ashei)* is a deciduous small tree most noted for its large 1–2 foot long leaves and fragrant creamy white flowers. Although the species is adapted to and used in landscapes in many parts of the U.S., it is endemic only to Northwest Florida where it is limited to ten counties growing on undisturbed bluffs and ravine banks. The populations are highly fragmented and are threatened by degradation of habitat, leading the species to be listed as endangered in the state of Florida. SSR markers were developed to determine the genetic diversity of wild populations of *M. ashei* in order to guide long-term conservation strategies. 18 marker loci identified a total of 82 alleles that were used to characterize allelic diversity of *M. ashei* from 11 wild populations, 14 cultivated sources, five accessions of *M. macrophylla*, and three interspecific hybrids. Results indicated a higher than expected level of heterozygosity within populations, and a clear distinction between Eastern and Western populations; conservation efforts should therefore focus on maintaining these distinct groups in corresponding ex situ seed orchards to counteract pressures due to overcollection, pollution, and loss of habitat due to development. Clustering of individuals was similar using several analytical methods, indicating that despite relatively small sample sizes, our analysis is a valid reflection of the diversity among and relationships between these populations.

Keywords Ashe magnolia · Conservation · Endangered plant · Genetic resource · Germplasm · Microsatellite

Introduction

Magnolia ashei Weath. (syn. *Magnolia macrophylla* ssp. *ashei*) is a deciduous understory shrub or small tree, most noted for its large leaves that can be over two feet long and up to a foot wide. The large (12″-diameter) fragrant flowers are creamy white with a purple blotch at the inner base of 6–9 petals. It is both outcrossing and self-compatible; known pollinators include bees, primarily *Lassioglossum reticulatum*, and various beetles (Latimer 1994). Although similar in appearance to *M. macrophylla* Michx., the smaller stature of *M. ashei* favors it for smaller commercial and residential landscape settings. It is also more floriferous than *M. macrophylla* and has a short juvenility period of only 2–3 years, making it a valuable plant for the nursery trade.

Magnolia ashei is endemic only to Northwest Florida where it is limited to ten counties in the panhandle (Wunderlin et al. 2016; Kevin Conrad, USDA-ARS, personal observation), from Pensacola to Tallahassee, growing on undisturbed bluffs and ravine banks. Due to its habitat specificity, populations of *M. ashei* are highly fragmented and vary substantially in size and density. For example, the Okaloosa Bayhead population comprised a substantial percentage of the understory and contained easily over 75 individuals, while we found only six individuals in the Bruce Creek site in two linear miles of suitable forest (see Table 1 for relative population sizes). Degradation of suitable sites caused by recreation, pollution, vegetation destruction, and development further threatens this species; it is listed as vulnerable by the Red List of Magnoliaceae (Cicuzza et al. 2007), and endangered by the state of Florida (USDA-NRCS 2017).

The primary goal of conservation is to maintain the full scope of natural variation that exists within a species or a population, thus avoiding any reduction of genetic diversity that could decrease fitness over time. Maintaining the evolutionary potential of a species is becoming especially

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Table 1 Accessions of *M. ashei, M. macrophylla*, and hybrids used in this study

Sample abbreviation[a]	# Samples	Source/origin and approximate population size (in parentheses)
Bay	5	Cat Creek population, Bay Co., FL (10 plants)
Jac	5	Jackson Co. population, FL (15 + plants)
Wak	3	Wakulla Co. population, FL (25 + plants)
Bru	6	Bruce Creek population, Walton Co., FL (6 plants)
Hol	16	Holmes Creek population, Washington Co., FL (25 + plants)
Oka	20	Okaloosa Bayhead population, Eglin Air Force Base, Okaloosa Co., FL (75 + plants)
RB	6	Rock Bluff population, Leon Co., FL (15 + plants)
TNC	4	The Nature Conservancy, Liberty Co., FL (25 + plants)
TorE	11	Torreya State Park—eastern population, Liberty Co., FL (11 plants)
TorW	19	Torreya State Park—western population, Liberty Co., FL (19 plants)
Weav	10	Weaver Creek population, Santa Rosa Co., FL (10 + plants)
Cul-AA1	1	Arnold Arboretum, Boston, MA—from plant in Gladwyne, PA
Cul-AA2	1	Arnold Arboretum, Boston, MA—commercially sourced from Liberty Co., FL
Cul-ABG	1	Atlanta Botanical Garden, GA—unknown source
Cul-BBG	1	Brookside Gardens, Wheaton, MD—from commercial nursery in Tallahassee, FL
Cul-GH	1	Thurmont, MD—Gordon Hagen, unknown source
Cul-GP	1	Garret Park, MD—unknown source
Cul-JCR	1	J.C. Raulston Arboretum, Raleigh, NC, via Mike Dirr from Superior Trees
Cul-NS	1	Newberry St, Aiken, SC—Bob McCartney—unknown source
Cul-Red	1	Red's Rhodies, Sherwood, OR—unknown source
Cul-SCBG	1	South Carolina Botanic Garden, Clemson, SC—unknown source
Cul-ST	1	Superior Trees, Lee, FL—collected in Leon Co., FL
Cul-UDBG	1	Univ. of Delaware Botanic Gardens, Newark, DE—from Woodlander's Nursery
Cul-USNA	1	US National Arboretum, wild collected from Walton Co., FL—accession NA56801
Cul-Woodl	1	Woodlander's Nursery, Aiken, SC—unknown source
Mac-ABG	1	*M. macrophylla*, Atlanta Bot. Garden—wild collected from Cumberland Falls, KY
Mac-Esc	1	*M. macrophylla*, wild collected in 2015 along Conecuh River, Escambia Co., AL
Mac-USNA	1	U.S. National Arboretum, Washington, DC—accession NA76201
Mac-Wal	2	*M. macrophylla*, wild collected in 2015 on Haines Island, Monroe Co., AL
Hyb-FM	1	(*M. macrophylla* x *ashei)* from Fred Meyer to Woodlander's Nursery
Hyb-MerC	1	(*M. ashei* x *macrophylla*) x *ashei*—Meredith College, Raleigh, NC
Hyb-ST	1	(*M. macrophylla* x *ashei*), Superior Trees, Lee, FL—unknown source

[a]Accessions with no prefix are wild-collected *M. ashei*; accessions beginning with "Cul" are plants identified as *M. ashei* from cultivated sources; accessions beginning with "Mac" are *M. macrophylla*; and accessions beginning with "Hyb" are interspecific hybrids between *M. ashei* and *M. macrophylla*

important due to impacts of pollution, deforestation, and climate change (Ledig 1988). Currently, many gaps in conservation efforts for Magnoliaceae exist, and the diversity of most species is not currently well represented in ex-situ collections (Cires et al. 2013). Unlike many other angiosperms, seeds of most Magnoliaceae species are recalcitrant, rendering seed banks ineffective; aside from in situ preservation, ex situ living collections are the most viable means of conservation for these plants until cryopreservation techniques become a feasible option (Bonner 1990; Oldfield 2009). For this strategy to be effective, it is necessary to understand the genetic diversity of the populations to be conserved, and then ensure that as much of this diversity as possible is represented in the ex situ collection (Griffith et al. 2015).

Magnoliaceae exemplifies the need for conservation, as 112 of 245 currently recognized species are threatened with extinction (Cicuzza et al. 2007; Cires et al. 2013). Simple sequence repeat (SSR or microsatellite) markers have proven useful for guiding conservation efforts for many rare and endangered species of horticultural or medicinal interest, including *Phellodendron amurense* (Yang et al. 2016), *Betula chichibuensis* (Igarishi et al. 2017), *Origanum compactum* (Aboukhalid et al. 2017), and *Narcissus tortifolius* (Jiménez et al. 2017). Within Magnoliaceae, SSRs have been used to characterize genetic and population structure of

Magnolia acuminata, Magnolia obovata, Michelia maudiae, Magnolia stellata, Magnolia sieboldii ssp. *japonica, Magnolia tripetala, Magnolia sharpii* and *Magnolia schiedeana*, among others (Budd et al. 2015; Gilkison 2013; Isagi et al. 1999; Kikuchi and Isagi 2002; Newton et al. 2008; Sun et al. 2010; Ueno et al. 2005). We therefore chose microsatellite markers to assess the genetic diversity of *M. ashei*. The objective of this study was to evaluate the genetic diversity of wild populations of *M. ashei* in order to guide decisions about long-term conservation strategies. To do so, we developed and utilized SSR markers to characterize the genetic diversity of samples of *M. ashei* from 11 wild populations, 14 cultivated sources, five accessions of *M. macrophylla*, and three interspecific hybrids.

Materials and methods

Plant material

Leaf samples were collected from mature trees from wild populations of *M. ashei* during three trips to the Florida panhandle (2013, 2015, and 2017). Eleven populations were sampled, representing the populations generally recognized in nine counties. These included Rock Bluff in Leon County; Torreya State Park (two locations, east and west) in Liberty County; The Nature Conservancy in Liberty County; Holmes Creek in Washington County; Bruce Creek in Walton County; Okaloosa Bayhead in Okaloosa County; Weaver Creek in Santa Rosa County; Econfina Creek in Bay County; Smith Creek in Wakulla County; and Pittman Hill Road in Jackson County (Fig. 1). Although the species is reported to be in Gadsden County (Latimer 1994), we could not find any references to site information and were unable to locate these trees on accessible land, so therefore did not sample from this county. For smaller populations, we sampled all mature trees from that population that we could locate or access. For the larger populations (Wakulla Co., Holmes Creek, Okaloosa Bayhead, and the Nature Conservancy), sample numbers varied depending on access and collecting conditions (Table 1). Accessions of *M. ashei* from cultivated sources (public gardens and commercial nurseries, assumed to be the pure species) were included to determine how much of the species diversity is represented in cultivated material. Three wild-collected samples and two cultivated samples of *M. macrophylla*, along with three *M. ashei* × *M. macrophylla* hybrids of cultivated origin were included for comparison (Table 1).

DNA extraction

Three to five newly-emerging leaves from each sample were dried in silica gel for later use. DNA was extracted using a PowerPlant Pro DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's specifications with the following modifications: the addition of a small amount of garnet matrix (BIO 101 Inc. Vista, CA, USA) to aid in homogenization, inclusion of the optional phenolic separation solution, and an additional wash step prior to elution. A NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to quantify the extracted DNA.

SSR development and primer selection

For SSR development, DNA isolated from a *M. ashei* specimen growing at the U.S. National Arboretum (Accession 56801*H) was sent to ACGT, Inc. (Germantown, MD), where a genomic library was developed. Sequence data was then generated using Illumina MiSeq (Illumina, San Diego, CA), producing 14M 2 × 300 paired-end reads. Adapter and low quality sequences (< Q30) were trimmed, and reads shorter than 100 nucleotides were discarded using trim_galore and sickle [Babraham Bioinformatics, Cambridge, UK; and OMICtools (Henry et. al. 2014)]. The trimmed R1 and R2 reads with a minimum overlapping

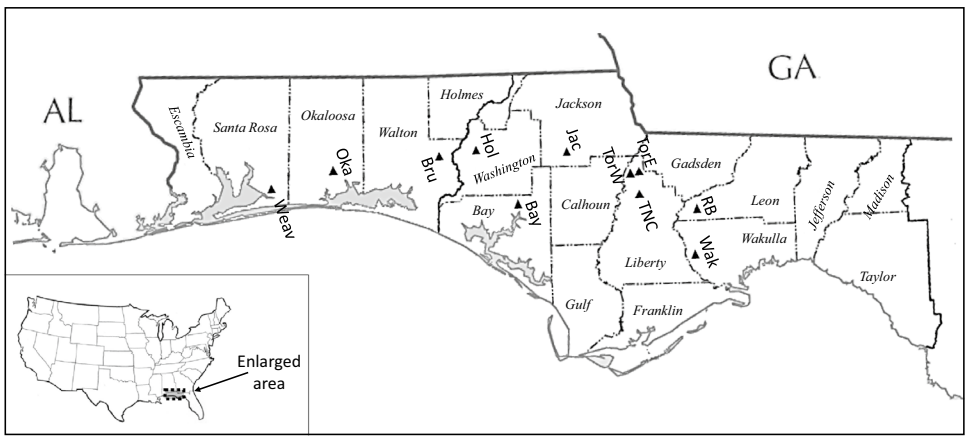

Fig. 1 Locations of populations of *M. ashei* in the panhandle region of Florida sampled for this study. All known populations were sampled except one possible population in Gadsden County

length of 15 bases were merged and assembled using CLC Genomics (CLC Bio, Waltham, MA), and contigs shorter than 500 base pairs were discarded. SSRs were identified using MISA (Beier et al. 2017), requiring a minimum repeat number of 6 for trinucleotide repeats, five for tetra-penta- hexa- and heptanucleotide repeats, and four for 8- and 9-base repeats. A total of 10,406 SSRs were identified. Tri-nucleotide and tetranucleotide repeats were the most prevalent with 7255 and 2399 occurrences, respectively. Dinucleotide repeats were excluded due to their greater tendency to show shadow bands (Chambers and MacAvoy 2000) and stuttering (Zalapa et al. 2012). SSRs with the greatest number of repeats were selected as they are expected to be more polymorphic than those with a smaller number of repeated units (Zalapa et al. 2012). The contigs containing SSRs were excluded if the flanking sequence either upstream or downstream from the microsatellite were of insufficient length for primer development.

Primers (Table 2) were developed using Primer 3 Plus (Untergasser et al. 2012). To the 5′ end of the forward primer, the M13 universal primer sequence (TGTAAA ACGACGGCCAGT) was added to allow for binding of a FAM-labeled M13 primer to the amplified product (Schuelke 2000). Three samples, one from each of three different *M. ashei* populations, were used to test for polymorphism and to identify optimum annealing temperatures for each primer using gradient PCR.

PCR amplification and scoring

PCR was carried out with a BioRad iCycler (Bio-Rad Laboratories, Hercules, CA, USA) in 15 µL reactions containing 30 ng of template genomic DNA, 1X MangoMix (Bioline Inc., Taunton, MA), 3.77 µM $MgCl_2$, 0.25 µM reverse and universal FAM-labeled M13 (-21) primer, and 0.0625 µM of the forward primer. The PCR amplification profile included an initial denaturation step at 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 s, 30 s at the optimized annealing temperature for each primer pair (Table 2), 60 s at 72 °C, and a final extension of 72 °C for 7 min. This profile was followed by eight cycles of 94 °C for 30 s, 53 °C for 45 s, and 72 °C for 45 s to allow annealing of the FAM-labeled M13 primer (Schuelke 2000). Amplified fragments were visualized on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using 1 µL PCR product, 10 µL HiDi formamide (Applied Biosystems) and 0.10 µL GeneScan 500 LIZ size standard (Applied Biosystems). Alleles were identified using Gene Marker version 2.6.3 (SoftGenetics, State College, PA, USA), with manual adjustments made according to Guichoux et al. (2011). A subset of samples, including any containing alleles with a frequency of < 5%, were repeated to ensure allele size scoring consistency. We also tested SSR primers previously developed for other Magnoliaceae species (Isagi et al. 1999; Jiang et al. 2011; Sun et al. 2010; Ueno et al. 2005; Yang et al. 2011; Zhao et al. 2012) using the methods described above.

Table 2 SSR primers developed and used in this study

Locus name	Core repeat	# Alleles	Allele size (bp)	Forward sequence	Reverse sequence	Ta (°C)
MA3-7	$(GAA)_{17}$	7	220–250	CATGCTAACCCATCTAGTCACG	TCCCAATACCCATCCCAGTA	56.3
MA3-12	$(GGA)_6$	2	201–204	AGCCCAAGGAGACAACAGAA	GGGTTTCTTCGCATGTTGTT	52.8
MA3-22	$(GCT)_7$	2	173–179	TCCAAGATTTCCTCGTCTGG	CGAGGGAGGAGTTCACGTAG	58.9
MA3-23	$(AGC)_9$	3	174–186	TCTTGCCGAAGTCAAAATGA	TACCGAATGCCATGAAAACA	54.0
MA3-27	$(TTC)_{14}$	6	220–257	ATCACCGATTTTAGCCTCCA	GACTGGCCCGTATGTTTGTC	58.1
MA3-28	$(TTC)_{14}$	8	218–237	TCGTTTTTCCATCAATATGCAG	TGCTGTTTTTCCACTGTGATTC	56.4
MA4-13	$(GAGT)_8$	3	253–258	TCATTCCCACCTTCTCATCC	GGCCCATCATATCAGCAGTT	54.1
MA4-16	$(AGTG)_7$	6	234–245	TGCGTGTGTGTTTGAGTGAG	TTAGAAAAGCGTGGCTGGTT	56.0
MA4-19	$(CCAT)_5$	2	264–272	TGGAAAGTGCACACTGGAAG	TTTCCATTAATCCGGGTCTG	55.9
MA5-5	$(GAAAG)_6$	4	296–312	AACGTTCAGCTTCTTGTTGGA	AGTCCAAGACCGAGCGAGT	57.4
MA5-12	$(CCCAA)_6$	7	144–179	TCATTTTCGATAGGGGACCA	AGTCGGACTTGGGTTGAGAA	58.3
MA5-13	$(ACCTT)_7$	2	188–203	GCTTGCATGCATATCACGTT	ACTCAGACCGGACGTAATGG	55.9
MA6-8	$(TGGGCT)_6$	5	166–196	GCTCGTGCACAAAAGAAGGT	CAGGCTGAGGTCTTTCCAAG	55.6
MA6-17	$(CCATCA)_5$	3	163–181	ATGCTGGAGGGATGAAACAT	TCTTCAGTGTTAGCCGCTCAT	57.0
MA6-18	$(CATCCT)_5$	3	236–254	CATCACCATCACCATCATCC	TAAGGCTCTCGCCAATAGGA	54.1
MA6-19	$(TGGTGC)_5$	4	209–228	GAGAAACCCTGCGAAAGAGA	ATGGTTAGCACCGAGCATTT	55.4
MA7-1	$(GAAAAAA)_5$	4	185–206	GTGGATATCATGGGCCTCAC	GGAGAAGTCCCTGCATGTGT	54.1
MA7-6	$(AAAAGAA)_5$	3	233–247	GGGAATGCCTCTAATGATGC	GGGTGAGTGAAACCTCTTGC	59.4

Data analysis

Gene frequency and population statistics (including identification of private alleles, number of effective alleles, Shannon's Index, observed and expected heterozygosity, and F_{is}, F_{st}, F_{it}, and Jost's index of differentiation, D_{est}) were calculated, and principal coordinate analysis was performed using GenAlex version 6.503 (Peakall and Smouse 2006) based on a diploid organism (Parris et al. 2010). Hardy–Weinberg tests were performed for each population at each locus in GenAlex to detect the presence of null alleles. Calculations were verified using GENEPOP (Raymond and Rousset 1995) and FSTAT (Goudet 2002), which was also used to calculate allelic richness. STRUCTURE was used to visualize population structure (Pritchard et al. 2000). We used default parameter settings, only systematically changing the number of groups, as suggested in Evanno et al. (2005), using code written in Perl to submit STRUCTURE calls in a loop and parse the output. STRUCTURE was run 20 times for each number of groups to estimate variability for that number of groups. Once the number of groups was determined, we averaged the posterior probabilities for each sample over the 20 runs. We then assigned each sample to a group if the posterior probability for the most predominant group was at least 0.75 (in most cases it was much closer to 1.00 than 0.75). For further analyses on each group, the few samples that could not be assigned this way (e.g. some hybrids) were included with any group for which they had a posterior probability of at least 0.25. NTSYSpc version 2.02 (Rohlf 1998) was used to visualize relationships between individuals based on genetic similarity (UPGMA using the Jaccard coefficient) and to develop a cophenetic correlation coefficient (r) using MXCOMP. Non-amplification at a locus was treated as missing data in GenAlex, and as absence of alleles in both STRUCTURE and NTSYS. The confidence levels for branches of the dendrogram were determined by calculating approximately unbiased (AU) *p*-values using multiscale bootstrap resampling based on 20,000 replications implemented in the 'pvclust' package version 1.3-2 of R software (Suzuki and Shimodaira 2014).

Results

When we used primers developed by other labs for other taxa in the Magnoliaceae, the amplification profiles for the primers tested were either monomorphic, or showed little or no amplification, non-specific amplification, or stutter bands (data not shown). Adjusting the magnesium concentration in the PCR reaction did not improve amplification, and reducing the denaturing temperature (Olejniczak and Krzyzosiak 2006) did not reduce the number or intensity of stutter bands. Therefore, we developed new microsatellite markers specifically for *M. ashei*. Of 64 primers that we developed and tested, 18 showed strong and specific amplification and were polymorphic. The remaining markers were either monomorphic, amplified poorly, or exhibited nonspecific amplification. The 18 SSR primer pairs identified a total of 72 alleles from the 105 wild *M. ashei* samples we tested, and a total of 82 alleles across all 127 accessions tested. The average number of alleles per locus was 4.1, ranging from 2 to 8 (Table 2).

Within each population, the observed heterozygosity (H_o) for each of the SSR loci ranged from 0.100 to 0.554, and the expected heterozygosity (H_e) ranged from 0.090 to 0.484. The heterozygote deficit, F_{is}, reflects the inbreeding coefficient within individuals relative to the subpopulation, and was not significant at $P = 0.10$ for any locus. D_{est}, an estimate of genetic differentiation based on the number of effective alleles, ranged from 0.059 to 0.572, with a mean of 0.263 (Table 3). Due to the small population sizes at Bruce Creek, Weaver Creek, and Rock Bluff, the assumptions necessary for Hardy–Weinberg equilibrium (HWE) were not met. Nevertheless, of the 198 tests performed (11 wild populations × 18 markers), only two showed significant deviation from HWE, having a heterozygote deficit, at $P < 0.01$—Bruce Creek for marker MA3-28, and Wakulla County for marker MA6-19. These factors suggest that these populations may have experienced a recent decline, supported by the lack of seedling recruitment we observed at these sites.

Populations differed in allelic richness, but not substantially so. Private alleles, defined as those found only in a single population, were identified in the Okaloosa Bayhead (5 private alleles), Weaver Creek (3), Bay County (3), Jackson County (3), Holmes Creek (2), Wakulla County (2), and Torreya West (1) populations. No private alleles were detected in the populations at Bruce Creek, Rock Bluff, Torreya East, or the Nature Conservancy from the samples we collected (Table 4).

The average number of alleles per locus ranged from 1.33 in the Rock Bluff population to 2.50 in the population at Okaloosa Bayhead. These two populations also exhibited the lowest and highest values, respectively, for number of effective alleles, average expected and observed heterozygosity, and Shannon's index (Table 4). AMOVA results, for the subset of our data containing all wild *M. ashei* samples and excluding any cultivated, hybrid, or *M. macrophylla* samples (Table 5) indicate that the majority (58%) of the genetic diversity of *M. ashei* exists within individuals. 39% of the variation is found among populations, while the remaining 3% of the variation is among individuals; F_{st} and F_{it} are significant at $P < 0.001$, and F_{is} is significant at $P < 0.03$.

We found 10 alleles from 7 loci that were present in *M. macrophylla* and *M. ashei-macrophylla* hybrids, but not present in wild populations of *M. ashei*. One or more of these alleles was also detected in three cultivated accessions

Table 3 Summary of diversity statistics for the 18 SSR loci across eleven wild *M. ashei* populations

Locus	N_a	N_e	I	H_o	H_e	μH_e	F	F_{is}	F_{it}	F_{st}	D_{est}	D_{est} prob.
MA3-7	2.818 (0.296)	2.123 (0.180)	0.815 (0.101)	0.513 (0.056)	0.484 (0.056)	0.527 (0.058)	−0.083 (0.058)	−0.059	0.316	0.355	0.520	0.000
MA3-12	1.273 (0.141)	1.248 (0.129)	0.182 (0.094)	0.107 (0.057)	0.130 (0.067)	0.139 (0.072)	0.179 (0.049)	0.174	0.544	0.447	0.119	0.003
MA3-22	1.364 (0.152)	1.210 (0.094)	0.196 (0.084)	0.139 (0.060)	0.130 (0.057)	0.137 (0.059)	−0.073 (0.033)	−0.065	0.378	0.416	0.106	0.003
MA3-23	1.727 (0.195)	1.373 (0.148)	0.331 (0.101)	0.200 (0.062)	0.206 (0.064)	0.219 (0.069)	−0.011 (0.078)	0.030	0.370	0.350	0.133	0.003
MA3-27	2.182 (0.352)	1.624 (0.183)	0.500 (0.132)	0.287 (0.094)	0.299 (0.078)	0.327 (0.084)	0.091 (0.144)	0.042	0.541	0.521	0.489	0.000
MA3-28	2.364 (0.244)	1.715 (0.159)	0.593 (0.110)	0.386 (0.082)	0.356 (0.067)	0.386 (0.074)	−0.080 (0.074)	−0.082	0.505	0.543	0.572	0.000
MA4-13	1.455 (0.157)	1.229 (0.095)	0.224 (0.082)	0.155 (0.065)	0.145 (0.055)	0.160 (0.062)	−0.052 (0.101)	−0.073	0.322	0.368	0.096	0.001
MA4-16	1.545 (0.207)	1.193 (0.086)	0.201 (0.079)	0.153 (0.068)	0.125 (0.052)	0.137 (0.058)	−0.169 (0.061)	−0.223	0.455	0.554	0.186	0.000
MA4-19	1.909 (0.091)	1.537 (0.116)	0.466 (0.069)	0.372 (0.076)	0.310 (0.053)	0.332 (0.057)	−0.181 (0.083)	−0.202	0.245	0.372	0.269	0.000
MA5-5	2.000 (0.357)	1.418 (0.125)	0.391 (0.118)	0.207 (0.068)	0.234 (0.069)	0.249 (0.073)	0.101 (0.118)	0.118	0.646	0.599	0.487	0.000
MA5-12	2.545 (0.247)	1.670 (0.118)	0.621 (0.072)	0.368 (0.067)	0.372 (0.041)	0.405 (0.045)	−0.015 (0.130)	0.012	0.468	0.461	0.533	0.000
MA5-13	1.545 (0.157)	1.368 (0.125)	0.307 (0.095)	0.206 (0.068)	0.209 (0.066)	0.224 (0.072)	0.013 (0.045)	0.013	0.498	0.491	0.263	0.000
MA6-8	2.000 (0.302)	1.569 (0.231)	0.436 (0.131)	0.263 (0.080)	0.260 (0.076)	0.284 (0.083)	−0.027 (0.077)	−0.009	0.475	0.480	0.336	0.000
MA6-17	1.273 (0.141)	1.152 (0.094)	0.138 (0.075)	0.100 (0.056)	0.090 (0.051)	0.095 (0.054)	−0.124 (0.036)	−0.106	0.312	0.378	0.059	0.004
MA6-18	1.636 (0.203)	1.242 (0.090)	0.255 (0.081)	0.156 (0.051)	0.157 (0.053)	0.165 (0.054)	−0.021 (0.032)	0.009	0.411	0.406	0.125	0.004
MA6-19	2.091 (0.163)	1.840 (0.118)	0.632 (0.076)	0.554 (0.078)	0.426 (0.049)	0.462 (0.053)	−0.299 (0.102)	−0.299	0.035	0.257	0.247	0.000
MA7-1	2.545 (0.282)	2.125 (0.224)	0.753 (0.129)	0.469 (0.090)	0.454 (0.075)	0.487 (0.081)	−0.028 (0.081)	−0.033	0.272	0.296	0.334	0.000
MA7-6	1.909 (0.211)	1.624 (0.204)	0.450 (0.118)	0.288 (0.090)	0.290 (0.078)	0.307 (0.082)	0.029 (0.102)	0.005	0.397	0.394	0.264	0.000

N_a mean number of alleles per population, N_e mean number of effective alleles per population, H_o observed heterozygosity, H_e expected heterozygosity, μHE unbiased expected heterozygosity, F mean Inbreeding coefficient, F_{is} inbreeding coefficient within individuals relative to the subpopulation, F_{it} inbreeding coefficient within individuals relative to the total, F_{st} inbreeding coefficient within subpopulations relative to the total, D_{est} Jost's estimate of differentiation. Standard deviations are noted in parentheses

Table 4 Summary of genetic diversity statistics for 11 populations of *M. ashei*

Population	N	Private alleles	A	A_r	A_e	H_e	H_o	F_{st}	I
Bay	5	3	2.111	1.744	1.766	0.328	0.375	−0.141	0.535
Jac	5	3	2.000	1.590	1.523	0.256	0.292	−0.128	0.429
Wak	3	2	1.389	1.322	1.231	0.141	0.157	−0.133	0.212
Bru	6	0	1.722	1.437	1.357	0.211	0.231	−0.081	0.335
Hol	16	2	2.222	1.725	1.744	0.361	0.375	−0.047	0.578
Oka	20	5	2.500	1.752	1.757	0.377	0.386	−0.053	0.617
RB	6	0	1.333	1.231	1.225	0.110	0.102	0.038	0.172
TNC	4	0	1.722	1.482	1.407	0.220	0.264	−0.189	0.347
TorE	11	0	1.889	1.497	1.489	0.248	0.237	0.024	0.396
TorW	19	1	2.111	1.555	1.502	0.282	0.275	0.049	0.461
Weav	10	3	1.889	1.644	1.659	0.325	0.313	0.035	0.495

See Table 1 and Fig. 1 for population names and locations

N total number of samples, *private Alleles* alleles unique to this population, *A* average number of alleles per locus, A_r allelic richness for 2 diploid individuals (2 rather than 3 based on missing data/nonamplification at one locus for a sample in one population), A_e Average number of effective alleles, H_e expected heterozygosity, H_o observed heterozygosity, F_{st} Wright's fixation index, *I* Shannon's index

Table 5 Analysis of molecular variance (AMOVA) from 11 wild populations of *M. ashei*

Source	df	SS	MS	Est. Var.	%	F-statistic	Value	P-value
Among populations	10	360.476	36.048	1.800	39%	F_{st}	0.388	0.000
Among invididuals	94	281.434	2.994	0.157	3%	F_{is}	0.055	0.021
Within individuals	105	281.500	2.681	2.681	58%	F_{it}	0.422	0.000
Total	209	923.410		4.638	100%			

(Cul-GH, Cul-NS, and Cul-ABG), raising questions as to the true species identity of those cultivated plants. *M. ashei* has been classified as its own species (e.g. Figlar and Nooteboom 2004; USDA-ARS 2017; Weatherby 1926) or as a subspecies of *M. macrophylla* (e.g. Figlar 1997; Kim et al. 2001; Spongberg 1976). While our results suggest a genetic distinction between *M. macrophylla* and wild populations of *M. ashei*, more taxonomic research is needed to determine whether, in fact, *M. ashei* is a species fully distinct from *M. macrophylla*.

Our Bayesian approach to population analysis using STRUCTURE software included wild-collected samples of *M. ashei* and *M. macrophylla*, cultivated *M. ashei* accessions, and hybrids between the two species. When all samples were analyzed together, STRUCTURE determined two populations (K = 2; Fig. 2) that correlate with the western and eastern populations. The wild-collected *M. ashei* samples from Okaloosa Bayhead, Weaver Creek, Bruce Creek, and Holmes Creek formed one subset (Western group), while accessions from the Nature Conservancy, Rock Bluff, Torreya, and Wakulla County formed another (Eastern group). All samples of *Magnolia macrophylla* and the three *M. ashei* × *M. macrophylla* hybrids fell into the Western group. Cultivated *M. ashei* samples were split between the groups. Twenty iterations of the STRUCTURE algorithm gave full consensus in the placement of samples within these groupings, with the exception of individuals from Jackson and Bay Counties, which didn't consistently fall into one group or the other. Perhaps not coincidentally, these two populations are located in the center of the range of this species, geographically between the two groups.

The two groups identified by the first STRUCTURE analysis were then analyzed separately. For the Western group (Okaloosa Bayhead, Weaver Creek, Bruce Creek, Holmes Creek, Jackson County, Bay County, *M. macrophylla*, and hybrids), STRUCTURE determined six populations (K = 6): Okaloosa Bayhead, Holmes, *M. macrophylla*, Bruce Creek, and Weaver Creek each formed groups, while Jackson and Bay County together formed a sixth group (Fig. 2a). In the Eastern group, Rock Bluff and Wakulla County was separated from the Torreya and Nature Conservancy populations (Fig. 2b).

A dendrogram generated using UPGMA clustering (Fig. 3) had relatively high bootstrap support and a cophenetic correlation coefficient (r) of 0.844, indicating a fairly good fit of clusters. The UPGMA analysis generated similar clusters as those identified by STRUCTURE software, but with several major distinctions. The UPGMA dendrogram identified four major groups, with the Holmes Creek population clustering the most distantly, but with only 82%

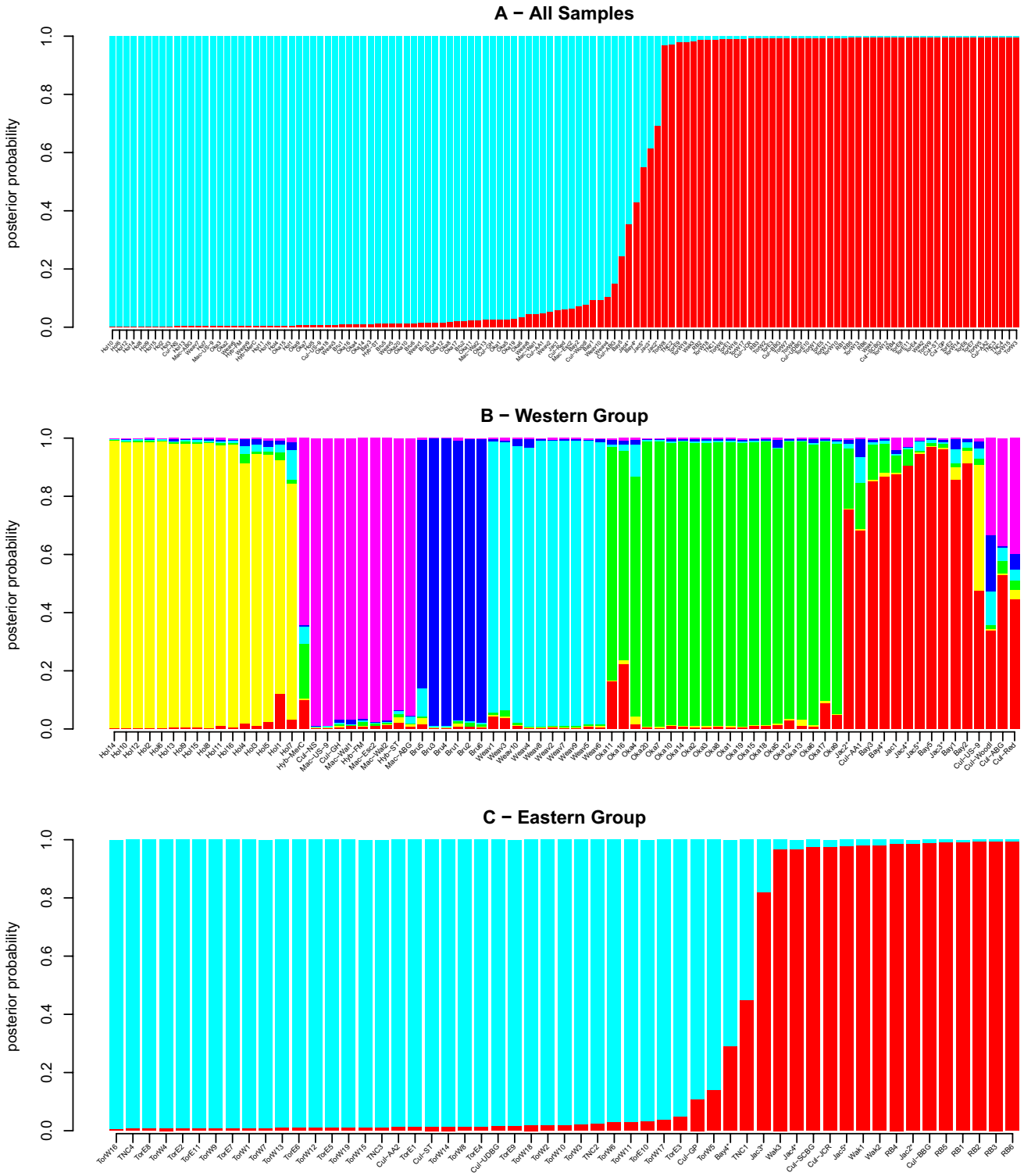

Fig. 2 Population structure of all 127 samples (**a**) and of the two subpopulations (**b, c**) identified by STRUCTURE analysis. Sample names are given along the x-axis. The Western subpopulation (K = 6) contains 77 accessions, and the Eastern subpopulation (K = 2) contains 55 accessions. Five accessions (Bay4, Jac2, Jac3, Jac4, and Jac5, indicated by asterisk on all plots) are included in both the Western and Eastern analyses because they did not clearly group with either of the two populations in the whole group analysis

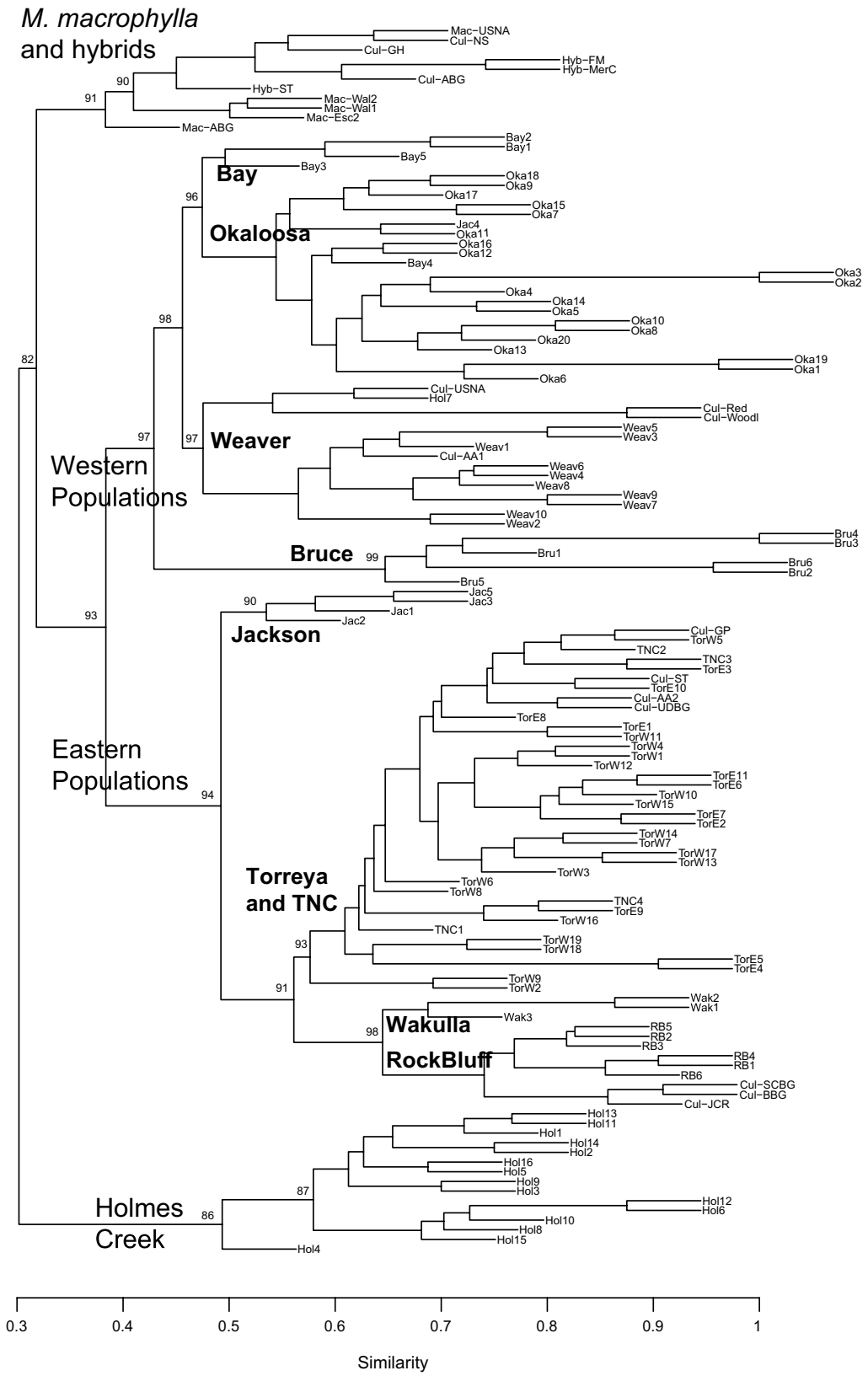

Fig. 3 UPGMA dendrogram showing clustering among all 127 samples based on Jaccard's similarity. Bootstrap values are shown above major nodes. Cophenetic correlation coefficient (*r*) is 0.844. Nodes are labelled with the predominant population represented in the cluster (Fig. 1; Table 1)

bootstrap support at this node (Cluster A in Fig. 3). The *M. macrophylla* and hybrid samples formed another cluster (Cluster D in Fig. 3), with 93% bootstrap support at that node. The remaining *M. ashei* samples were separated into two well-supported clusters (B and C in Fig. 3). While STRUCTURE broke the populations into similar groups, the relationship of these clusters to each other differed between the two analyses. UPGMA Cluster B (with the exception of the AJC population) formed one STRUCTURE population (Fig. 2b), and all the remaining UPGMA clusters (A, C, D) formed the other larger STRUCTURE population (Fig. 2a). When the two large STRUCTURE populations were broken down into smaller populations (K = 6 and K = 2), these populations followed the major clusters of the UPGMA dendrogram. The somewhat low bootstrap values at these nodes (82, 86, 91%), as well as the inability of STRUCTURE to definitively place several individuals into groups, reflects the fact that some of these populations share alleles and may not be as distinct as geography might suggest. Indistinct grouping in STRUCTURE or lower bootstrap values in the dendrogram could also be due to several samples that were missing particular loci altogether (number missing in parentheses). These were Bru2 (1), Weav3 (1), Bay3 (2), Bay4 (1), Jac2 (5), Wak1 (1), Cul-NS (1), Mac-Wal2 (2), and Mac-ABG (3).

Not unexpectedly, Principal Coordinates Analysis of individuals sampled from the wild populations revealed similar groups as those resulting from UPGMA clustering (Fig. 4). The first three components accounted for only 58.9% of the total variation, and it took nine components to explain 80% of the variation, so this method was not useful for reducing the dimensionality. Like the UPGMA clusters, the population from Holmes Creek appeared to be the most distantly related to the other populations and also had the most diversity among samples.

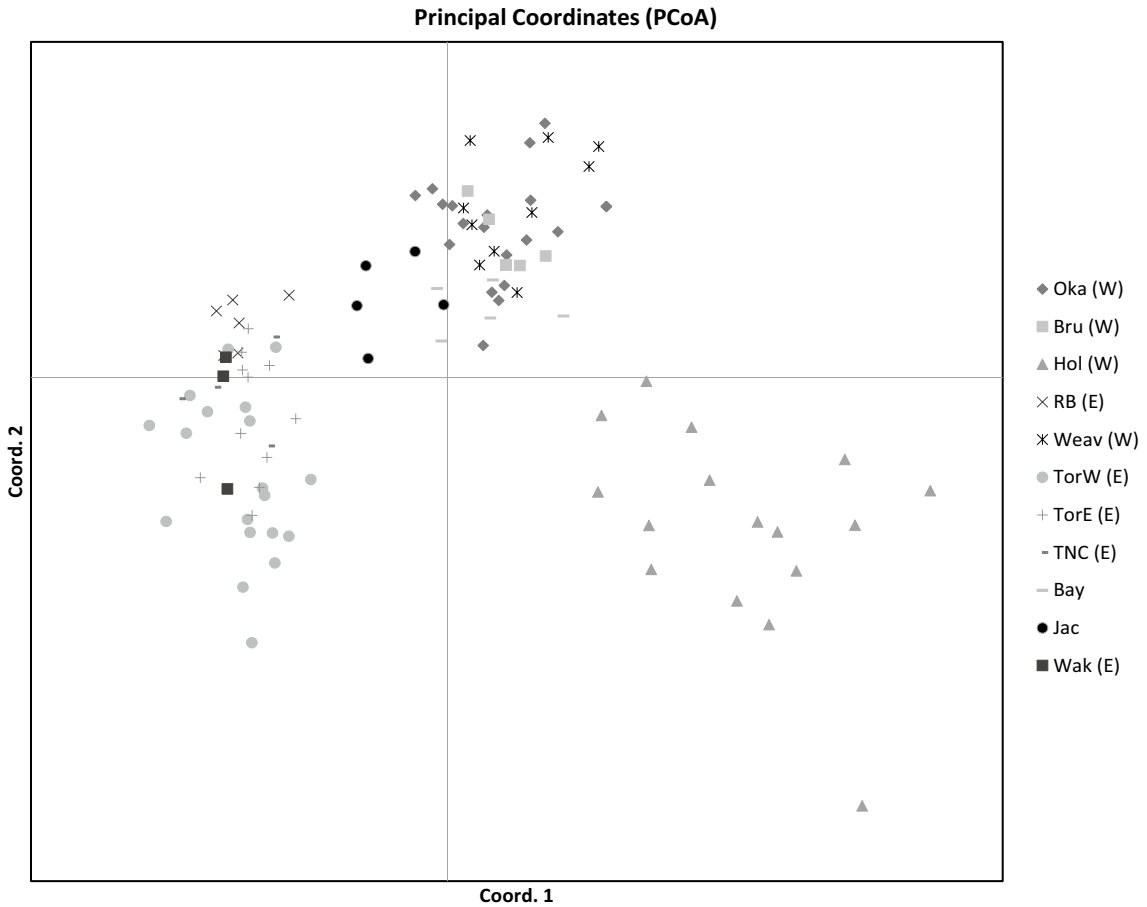

Fig. 4 Principal coordinates analysis of the 105 samples from wild populations of *M. ashei*. The first three components accounted for 37.8, 13.6 and 7.4% of the total variation. For cross-reference with Figs. 1, 2 and 3, relative locations (West or East) of each population are indicated beside the population name

Discussion

Likely owing to its small population sizes and fragmented distribution, a considerable portion (39%) of the genetic diversity found within *M. ashei* is partitioned among its various populations. This suggests that the geographic fragmentation of the populations is not due to recent over-exploitation or development as in *Michelia coriacea* (Zhao et al. 2012), but rather due to its evolutionary history. Miller (1975) suggested that the north-facing bluffs and ravine banks on which *M. ashei* grows may have offered protection for *M. ashei* and other associated species that are generally adapted to more northern climates but may no longer be competitive there. Like other magnolia species, *M. ashei* is thought to be pollinated by beetles, bees, and thrips (Thien et al. 1998), which may have also contributed to the population structure seen today.

We recognize the limitations of the small sample sizes we used to calculate population structure, and caution that the statistics reported in Table 3 (for primers) and especially Tables 4 and 5 (for populations) should be regarded with the caveat of these small numbers in mind. Ideally for studies of population structure and diversity, population sizes and range would be determined first, followed by methodical sampling from transects. However, for *M. ashei*, the locations and sizes of several populations are still uncertain; for several populations (Bay, Bru, TorE, TorW, and Weav), we sampled all the trees we could reasonably access. In some cases, practical aspects of sample collecting (e.g. access to land, safety concerns) limited our ability to sample more individuals. Despite the small sample sizes, the fact that samples from each population clustered together using multiple analyses (STRUCTURE, PCoA, UPGMA) indicates that these samples may accurately reflect the relationships among the populations, even if the population statistics are not as robust.

Although the molecular genetic differences between populations are statistically significant, it is possible that these differences do not have biological significance; in this case, the seemingly distinct differences between some populations could simply be due to the statistical power of the high degree of variability for the markers used (Hedrick 1999, 2001). Jost (2008) demonstrated that the use of highly variable markers results in an inaccurate measure of diversity/differentiation when relying on F_{st} or G_{st}; Bird et al. (2011) likewise recommended using D_{est} for datasets involving a greater number of alleles per locus. D_{est} is included for this reason. We recognize that there are many ways to define, measure, and analyze genetic diversity (Vane-Wright et al. 1991), and that population size and selection pressure cannot be used to predict population differentiation. For example, some tree species display little diversity among populations even with selection pressure (e.g. *Ulmus rubra*, Brunet et al. 2016; *Magnolia tripetala*; Gilkison 2013, *Juglans cinera*; Ross-Davis et al. 2008), whereas in other species, populations can be quite distinct (e.g. *Argania spinosa*, El-Mousadik and Petit 1996). Our results are consistent with previous studies by Latimer (1994) who found low levels of genetic variability within *M. ashei* populations and considerable genetic differentiation among populations. These population effects could be caused by a combination of historic bottlenecks, founder effects, geographic isolation of populations, and effects of pollination and reproductive biology. In addition, due to the nature of sampling (which generally tends to favor individuals that are easily located or accessible, and near each other), within population diversity is likely underestimated.

The Global Strategy for Plant Conservation, adopted as part of the Convention on Biological Diversity in 2002, includes 16 targets to prevent the loss of plant diversity. Relevant to this study, the goal of Target #8 is to conserve at least 75% of the world's threatened plants in ex situ collections by 2020 (Hird and Kramer 2013). Genetic data, such as that generated by this study, should be used to ensure that genetic and allelic diversity is captured or conserved using the fewest number of populations (Neel and Cummings 2003). Such an approach has been used in numerous other studies to facilitate selection of plants for conservation. For example, Ceska et al. (1997) used allozymes in *Baptisia arachnifera* to determine which and how many populations should be sampled to capture 99% of the diversity present. Data on the distribution of genetic variation in *Antirrhinum microphyllum* (Torres et al. 2003) and *Argania spinosa* (El-Mousadik and Petit 1996) illustrated the presence of and need to conserve private alleles in those populations. Other studies have shown the value of marker-assisted methods to establish or maintain germplasm collections (Bataillon et al. 1996; Schoen and Brown 1993). Management of protected populations in situ, in which seeds are harvested, germinated under controlled conditions, and replanted at random has shown promise for northern populations of *Magnolia acuminata* (Budd et al. 2015).

Based on our observations and those of others (Latimer 1994; Miller 1975), the native populations of *M. ashei* are generally stable, healthy, and well-distributed; however seedling recruitment and survival are quite low, both in the wild and in commercial nurseries. The low recruitment rate could be due to lack of viable seed production, seed predation, an inhospitable seed germination environment, or genetic factors due to incompatibility or inbreeding. Reduced seed germination has also been observed in a controlled propagation environment using cultivated accessions of *M. ashei* (Smit et al. 2016). One of the current threats to *M. ashei* is the possible over-collection of seeds for the nursery industry

from some populations, which further reduces the ability of native populations to regenerate. Collecting a small number of seeds from a large number of individuals can effectively conserve high levels of variability while simultaneously avoiding a significant reduction in seedling recruitment (Jiménez et al. 2017). The lack of younger plants at some sites indicates that these populations are currently not self-sustaining and that ex situ conservation is likely necessary for long-term preservation of the diversity of this species. We therefore propose that the best strategy for conserving the diversity of *M. ashei* is to create multiple seed orchards for genetically distinct populations. The implementation of seed orchards would eliminate the need for collecting seed from wild populations and would conserve material for potential future reintroduction or augmentation of existing populations. Since the majority of the genetic diversity in the populations we studied occurs within individuals (58%), seed orchards comprising a greater number of individuals would be more efficient than ensuring each population is maintained in its own respective seed orchard. Maintaining a small number of seed orchards will ensure that at least a portion of the 39% among population diversity will be captured. Ideally, a seed orchard would be established for each population; however resources may not allow for such a strategy. At a minimum, two seed orchards should be created, with one containing individuals from Okaloosa, Bruce Creek, Holmes Creek, and Weaver Creek, and the other representing Torreya, the Nature Conservancy, and Rock Bluff. If resources allow for the creation of a third, that seed orchard should represent the Holmes Creek population.

Our study and a survey by Smit et al. (2016), indicates that accessions of *M. ashei* in cultivation are derived from only a few of the natural populations we sampled. Botanic gardens and commercial nurseries could therefore play an important role in conserving this species by deliberate selection and planting of trees representing known provenances. Sixteen botanic institutions have expressed interest in becoming involved with such conservation efforts (Smit et al. 2016); we hope that this study will provide the necessary data to embark on this worthwhile endeavor.n.

While our primary objective was to evaluate the genetic diversity of wild populations of *M. ashei* in order to guide decisions about long-term conservation strategies, we also learned valuable information about the identity and diversity of cultivated *M. ashei*. While the cultivated forms are generally diverse, as seen in Figs. 2 and 3, they clearly do not represent all of the wild populations. Specifically, it appears that alleles from Bruce Creek, Holmes Creek, Okaloosa Bayhead, and Weaver Creek are not represented in the cultivated material that we tested. Therefore, the results from this study can also be used to inform decisions about which seed orchards to choose from in order to maximize the genetic base of cultivated *M. ashei* in a landscape.

Our study provides a detailed look at the population structure of *M. ashei* from much of its native range, and also provides new tools, in the form of SSR markers, for further evaluation of genetic diversity for conservation of this species. Geographic and climatic data is being analyzed to predict additional sites that may contain as yet undiscovered populations of *M. ashei* (Gary Knox, University of Florida, personal communication), as protection of these plants in situ is generally the most effective conservation method (Chazdon and Laestadius 2016; Ellstrand and Elam 1993; Riggs 1990). In addition to establishing ex-situ seed orchards, there may still be opportunities to conserve allelic diversity in situ by habitat preservation and possibly using controlled interbreeding among populations to maximize allelic richness. However, because some of the populations are small and isolated, additional research using larger sample sizes is necessary to determine the effects of genetic drift, inbreeding, and gene flow.

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