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Inter Simple Sequence Repeats (ISSR) Reveal Genetic Variation Among Mid-Atlantic Populations of Threatened *Amaranthus pumilus* and Phylogenetic Relationships

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ABSTRACT *Amaranthus* (Amaranthaceae) is a globally distributed plant genus composed of both weedy and cultivated species. While there have been previous attempts to resolve phylogenetic relationships within the genus, little attention has been placed on systematic relationships of the federally threatened coastal species *Amaranthus pumilus* Raf., endemic to eastern United States barrier islands, nor on genetic variability within the genus. In the present study, single primer ISSRs were employed to measure both genetic diversity and the phylogenetic position of *A. pumilus*. Leaf tissue samples were taken from wild populations on Fenwick Island, Delaware and from wild and propagated populations on Assateague Island National Seashore, Maryland. Genetic variation was detected among and within *A. pumilus* populations, though variability was low. Fenwick populations exhibited the highest genetic variability (h = 0.1016), while on Assateague the wild *A. pumilus* population had higher variability (0.0340) than the propagated population (0.0185). Due to its desirable characteristics in plant breeding trials, genetic variation within *A. pumilus* was also compared to variation of grain varieties *A. hypochondriacus* L. and *A. cruentus* L. Genetic diversity within *A. pumilus* was lower than either grain species sampled (0.2263 and 0.2947). Phylogenetic analyses included 41 accessions representing 33 *Amaranthus* species, and maximum parsimony, neighbor-joining, and Bayesian consensus trees were constructed. Though considerable phylogenetic signal was detected within the data matrix, phylogenetic resolution was low. *Amaranthus pumilus* grouped with the coastal species *A. arenicola* I.M. Johnst. in all consensus trees, which is the first postulated relationship of this pair.

INTRODUCTION Inter simple sequence repeats (ISSRs) have been widely recognized as a fast and effective marker in genetic fingerprinting, phylogenetic analyses and in studies of genetic diversity (Bussell et al. 2005). ISSRs are highly variable sequences of DNA that are flanked on either side by a series of tandem di- or trinucleotide simple sequence repeats (microsatellites). The primers used in the ISSR technique are complementary to the microsatellite sites and bind to the end of each site with a 1–3 nucleotide anchor sequence at either the 5′ or 3′ end. Thus, the ISSR region between two neighboring microsatellite sites is amplified (Culley and Wolfe 2001, Rakoczy-Trojanowska and Bolibok 2004). By screening primers that are complements to microsatellite repeats, sequencing is not required for the ISSR technique and it is

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rapid as well as cost-effective (Alhani and Wilkinson 2006). The single-primer PCR method generates ISSR markers that are typically inherited in a dominant Mendelian fashion (Van der Nest et al. 2008).

The utility of ISSRs in studies of genetic diversity and their subsequent utility in detecting polymorphisms within plant populations have been well demonstrated. Rodríguez-Echeverría et al. (2008) illustrated the effectiveness of ISSRs by revealing genetic diversity within and between populations of the coastal species *Ammophila arenaria* L. Spagnuolo et al. (2007) evaluated genetic variation of *Pleurochaete squarrosa* Brid. using ISSRs while Goldman (2008) reported the reproducibility of ISSR polymorphisms and success measuring genetic diversity analyses of Texas bluegrass hybrid species. In addition to their practicality, ISSRs rival other arbitrarily amplified PCR-based markers such as RAPDs and AFLPs in their ability to detect polymorphisms (Galvan et al. 2003, Nybom 2004, Praveen et al. 2009).

The species relationships within the plant genus *Amaranthus* may be better understood from the application of ISSRs. Though several studies have investigated phylogenetic relationships and genetic diversity within *Amaranthus* (Lanoue et al. 1996, Chan and Sun 1997, Marcone 2000, Xu and Sun 2001), most reflect either small portions of the genus or focus on the economically significant grain species while excluding other species.

Amaranthus (Amaranthaceae) is composed of over sixty morphologically diverse species (Sauer 1967, 1976; Steckel 2007) that are globally distributed and represent both wild (common weed) and grain varieties. The grain varieties of amaranth (particularly *A. hypochondriacus* L., *A. cruentus* L. and *A. caudatus* L.) may be excellent candidates for future plant breeding trials. Of the weedy amaranth cultivars, *Amaranthus pumilus* (seabeach amaranth) may also prove advantageous in plant breeding programs because of its drought tolerance, ability to thrive in poor soil conditions, and its production of highly fertile, large-sized seeds (Marcone 2000).

Amaranthus pumilus is a federally threatened plant species endemic to the Atlantic coast barrier islands of the United States. Historically ranging from Massachusetts to South Carolina, this species has been extirpated from three-fourths of its historic distribution (Weakley et al. 1996, Hancock and Hosier 2003). *A. pumilus* opportunistically inhabits wrack-lines and overwash flats along lower foredune regions, allowing *A. pumilus* to colonize available habitat as a pioneer species following abiotic disturbances (Feagin et al. 2005). *Amaranthus pumilus* had not been reported in Maryland since 1966 when in 1998, two plants were discovered during a floristic survey of Assateague Island National Seashore by National Park Service and Maryland Department of Natural Resources staff. This represented the first documented occurrence of *A. pumilus* between North Carolina and New York since 1972 and the first on Assateague Island in 31 years (Weakley et al. 1996, Lea and King 2001). Curiously, in 2000, a wild *A. pumilus* population (50 individuals) was discovered on nearby Fenwick Island, Delaware (nearly 56 kilometers north of Assateague Island). The species was last recorded on Fenwick Island in 1875 (Lea and King 2001). The discovery of the two *A. pumilus* individuals on Assateague prompted a restoration program led by the National Park Service. One of the two plants was salvaged and propagated with the ultimate goal of restoring the progeny. Despite this reemergence, few studies have investigated the genetic diversity of *A. pumilus* populations found in the Mid-Atlantic region.

The aim of the present study was to (1) employ ISSRs to measure genetic variability among and within both wild and propagated Mid-Atlantic *A. pumilus* populations (Assateague and Fenwick Islands), (2) compare genetic diversity of *A. pumilus* to the cultivated amaranth grain varieties (*A. hypochondriacus* and *A. cruentus*), and (3) examine phylogenetic relationships within *Amaranthus* (including grain and weedy species) with special emphasis on *A. pumilus*. To our knowledge, this is the largest phylogenetic study of *Amaranthus* to incorporate *A. pumilus* using single-primer ISSR markers.

METHODS

Plant Material

Leaf tissue samples from 33 *Amaranthus pumilus* specimens were collected from two Mid-Atlantic barrier islands, Assateague Is-

land, MD (37°58′N, 75°18′W) and Fenwick Island, DE (38°27′33.53″N, 75°3′12.83″W). On Assateague Island, 12 transplanted *A. pumilus* plants along with 4 wild individuals were sampled for genetic variation measurements. The transplanted plants were the second filial progeny of the original salvaged *A. pumilus* individual (the origin of the individual plants was confirmed based on recorded planting locations of the transplanted individuals). Leaf tissue from 17 wild *A. pumilus* individuals was collected from Fenwick Island. Specimens were sampled throughout the islands to reduce the likelihood of sampling clones from the parental generation of this selfing species. Seeds of the remaining amaranth species used in the phylogenetic analyses were obtained from the United States Department of Agriculture, Agricultural Research Services Plant Introduction Station in Ames, Iowa (Table 1) and grown in a greenhouse at Salisbury University, Maryland. In total, 33 *Amaranthus* species were included in the phylogenetic analysis.

ISSR-PCR Amplification

Total genomic DNA (gDNA) was extracted using Qiagen's (Chatsworth, California) DNeasy Plant Extraction Kit following the manufacturer's protocols. Subsets of leaf tissue from the *A. pumilus* were used to screen 50 ISSR primers obtained from the University of British Columbia Biotechnology Laboratory. Five primers produced polymorphic bands and were subsequently used in the analysis of genetic diversity among and within *A. pumilus* populations: Primer Nos. 811 (GA_8C), 840 (GA_8YT), 842 (GA_8A), 846 (CA_8RT), and 855 (AC_8YT), where R=A,G; Y=C,T. For comparison of interspecific variability among *A. cruentus*, *A. hypochondriacus*, and *A. pumilus* and for phylogenetic analyses, the same 50 ISSR primers obtained from the University of British Columbia Biotechnology Laboratory were screened and 7 produced consistent polymorphic bands: 807 (AG_8T), 811, 812 (GA_8A), 820 (GT_8C), 822 (TC_8A), 840 and 899 (CAT TCC CCA CAG GTT AAC ACA). PCR mixtures were carried out in 25 μL volumes consisting of 18.3 μL $_dH_2O$, 2.5 μL Stratagene Mastermix, 1.5 μL primer, 1.5 μL DNA, 0.5 μL Stratagene Buffer 3, 0.5 μL dNTP and 0.2 μL *Taq*. An Eppendorf Mastercyler (Model 5331) was used for amplification with an initial denaturation period of 1.5 minutes at 94°C; 35 cycles of 40s at 94°C, 45s at 44°C, 1.5 minutes at 72°C and a final 5 min extension at 72°C; 4°C hold.

Electrophoresis and Data Analysis

The resulting PCR products were stained with ethidium bromide and characterized on 1.5% agarose gels in 1X Tris-Borate-EDTA (TBE) Buffer. ISSR bands were visualized under UV light, digitally documented and analyzed using BioMax 1D image analysis software (Eastman Kodak Company). Fragment sizes were estimated based on 1-kb ladder size standards according to the BioMax 1D software algorithm. All Bands were interpreted as dominant markers and were visually scored as diallelic regardless of band intensity.

Genetic Variation Analysis

Haplotypes, allele frequency, percentage of polymorphic loci and heterozygosity were calculated for *A. pumilus* populations using Tools for Population Genetic Analysis (TFPGA) 1.3 (Nei 1972, Miller 1997). Analysis of Molecular Variance (AMOVA) was performed using AMOVA 1.55 (Excoffier et al. 1992) to measure variation within and among *A. pumilus* populations. The POPGENE software program (Yeh et al. 1997) was used to measure genetic diversity.

Phylogenetic Analysis

A Bayesian tree was constructed (Figure 1) using the software program Mr. Bayes (Huelsenbeck et al. 2001, Ronquist and Huelsenbeck 2003) set to following parameters: nst=6, rates=gamma, ngen=1,000,000, printfreq=1,000, samplefreq=100, nchains=4, burnin=1,000. Additionally, a maximum parsimony consensus tree and a neighbor-joining tree were generated using PAUP* 4.0b10 (Swofford 1998) using the heuristic search option. Tree support for maximum parsimony and neighbor-joining trees was assessed by bootstrap analysis using 1,000 replicates and clades with a frequency of >50% were retained. Starting trees were obtained via stepwise addition using a simple addition sequence. The tree-bisection-reconstruction (TBR) technique was employed to resample relationships detected under the parsimonious criteria while the neighbor-joining BIONJ algorithm was applied to the neighbor-joining

Table 1. Species and accessions of *Amaranthus* obtained from the United State Department of Agriculture (Ames, Iowa)

Species	Source	Accession
A. acutilobus Uline Bray	Germany (2)	AMES 13786, 13787
A. aff. blitum	Kenya	PI 490298
	Bangladesh	PI 606282
A. arenicola I.M. Johnst.	U.S. Kansas	PI 607459
A. asplundii Thell.	Ecuador	PI 604196
A. australis Sauer	Florida (2)	PI 553076, 553076
A. blitoides S. Wats.	Iowa	PI 553079
	Canada	PI 608663
A. blitum L.	India (2)	PI 288277, 608661
A. californicus S. Wats.	U.S. California	PI 595319
A. cannabinus Sauer	U.S. Virginia	PI 568124
A. caudatus L.	Ecuador	PI 490609
	Peru	PI 490642
A. crassipes Schlecht.	Czechoslovakia	AMES 10339
A. crispus N. Terracc.	Hungary, Hajdu-Bihar	AMES 21715
A. cruentus L.	Guatemala	AMES 5142
	India, Kerela	PI 566897
	Mexico, Morelos (2)	AMES 5171, 5493
	Mexico, Puebla	AMES 5638
	Mexico, Sonora (2)	AMES 5310, 5648
	Zaire	AMES 5369
A. deflexus L.	Portugal	AMES 13779
A. fimbriatus S. Wats.	Mexico, Sonora	PI 605738
A. floridanus Sauer	U.S. Florida	PI 553078
A. dubius Mart.	Jamaica	PI 605738
A. graecizans silvestris O. Bolòs & Vigo	Portugal, Coimbra	AMES 24671
A. Hybrid	U.S. Pennsylvania (2)	PI 538323, 538324
A. hypochondriacus L.	India (3)	PI 274279, 477916, 481134
	Mexico	PI 477917
	Mexico, Chihuahua (2)	AMES 5132, 5321
	Mexico, Oaxaca	AMES 5467
A. muricatus Gillies	Spain	AMES 21716
A. palmeri S. Wats.	U.S. Arizona	AMES 5370
A. powellii S. Wats.	Germany	PI 572261
A. pumilus Raff.	ASIS*	(wild specimen)
A. quitensis Kunth	Ecuador	PI 511745
A. retroflexus L.	U.S. Iowa	PI 572263
	Jamaica	PI 607447
A. rudis Sauer.	U.S. Nebraska	PI 603873
A. spinosus L.	Zimbabwe	PI 482057
A. standleyanus Parodi	Argentina, La Pampa	PI 605739
A. tricolor L.	U.S. Pennsylvania	PI 477918
	India, Tamil Nadu	PI 566899
A. tuberculatus Sauer	U.S. Indiana	PI 603881
A. viridis L.	Indonesia, Java	PI 540445

*ASIS = Assateague Island, Maryland.

tree. Phylogenetic signal was confirmed based on the G_1 statistic (Hillis and Huelsenbeck 1992) and skewness of tree length frequency distribution was estimated from 10^6 randomly generated parsimonious trees. Eight total replicates (*A. aff. blitum*, *A. tricolor*, *A. acutilobus*, *A. caudatus*, *A.* hybrid, *A. retroflexus*, *A. blitum* and *A. blitoides*) were included to access potential intraspecific variability of species. *Celosia cristata* L. (Amaranthaceae) and *Spinacia oleracea* L. (Chenopodiaceae) were selected as an outgroup based on their known genetic relationship to *Amaranthus* (Nath et al. 1992, Müller and Borsch 2005).

RESULTS

Genetic Variation

A total of 43 bands were scored for 33 *Amaranthus pumilus* individuals, of which 12

Table 2. Measures of genetic diversity in three populations of *Amaranthus pumilus*

Population	Haplotype	Heterozygosity	% Poly Loci	% Poly Loci/N
Assateague		0.0444	16.2791	1.0174
Propagated	B, C	0.0185	11.6279	0.9689
Wild	A, B	0.0340	9.3023	2.3256
Fenwick				
Wild	B, D, E, F	0.1016	25.5814	1.5048

were polymorphic. A ϕ-statistic indicated that there was a relatively weak positive association between the binary characters within our data matrix. Overall, genetic diversity values were low for all three *A. pumilus* populations (Table 2). The assumption of equal variances between populations on Assateague Island was rejected based on a Bartlett's statistic (Table 3). Analysis of Molecular Variance (AMOVA) revealed a statistically significant difference ($p < 0.0005$) of variances between wild and propagated populations on Assateague Island (32.78% among populations; 67.22% within populations). On Assateague Island, the wild population displayed a higher genetic diversity value than the restored population while the highest diversity value overall was detected in the Fenwick Island population (Table 2). Population level genetic diversity statistics are summarized in Tables 2 and 3. At the interspecific level, gene diversity (h, Table 4) was observable among grain varieties *A. hypochondriacus* and *A. cruentus* and among and between *A. pumilus* populations. Six haplotypes were identified with the following frequencies and distributions: Haplotype A was discovered in three of the four wild Assateague Island individuals. Haplotype B was found in the fourth wild individual along with the majority of the propagated samples on the same island and three individuals from Fenwick Island (14 total). Haplotype C was documented in two propagated *A. pumilus* individuals on Assateague Island while Haplotype D was found in 10 Fenwick Island individuals. Haplotype E was discovered in two Fenwick Island individuals while Haplotype F was found in one individual, also from Fenwick Island (Table 2).

Phylogenetic Analysis

A total of 114 bands of 33 amaranth species were scored with an average of 19 bands per primer. Amplified fragment sizes ranged from 299 to 3716 bp. A strict consensus parsimony tree (Figure 2) was generated from a total of 113 characters (104 of which were informative) and was poorly resolved. The neighbor-joining method also resulted in a poorly resolved tree (Figure 3). Phylogenetic signal within the data matrix was identified using the G_1 statistic (Hillis and Huelsenbeck 1992) and revealed a value of −0.201191 (mean = 976.3, standard deviation = 14.85). One million randomly generated parsimonious trees were constructed and tree length distribution frequency was left-skewed. Homoplasy within the binary data matrix was measured using PAUP and revealed a Homoplasy Index (HI) of 0.891 and a Consistency Index (CI) of 0.109. Tree robustness was assessed by bootstrap analysis using 1,000 replicates and only frequencies greater than 50% were reported. Additionally, a Bayesian consensus tree was constructed, but showed similarly ambiguous results (Figure 1). Overall, strong statistical support was seen in accessions of the same species. Multiple accessions of *A. australis* and *A. acutilobus* did not group together by their respective species. *A. pumilus* consistently grouped with *A. arenicola* in both neighbor-

Table 3. Analysis of Molecular Variance (AMOVA) of 33 *Amaranthus pumilus* wild and restored individuals on Assateague Island (within populations) and the wild Fenwick Island population

Source of Variation	*df*	Variance Component	% Total Variance	P-value *	ϕ-Statistic	Bartlett's Statistic
Among Populations	1	0.59	32.78	0.0005	0.328	0.05
Within Population	31	1.2	67.22	0.0005		

Table 4. Genetic variation among *Amaranthus pumilus* and two grain amaranth species

Species	Gene Diversity (h)
A. cruentus	0.2947
A. hypochondriacus	0.2263
A. pumilus:	
ASIS Propagated	0.0185
ASIS Wild	0.034
Fenwick (Wild)	0.1016

ASIS = Assateague Island, Maryland.

joining and Bayesian trees but did not group by parsimony.

DISCUSSION Knowledge of genetic variation in small populations of rare plant species is vital to conservation strategies (Schemske et al. 1994, Vergeer et al. 2003, Hensen and Oberprieler 2005). The independent efforts of the NPS to propagate threatened *Amaranthus pumilus* following its reemergence provided a unique opportunity to examine genetic variability between restored and wild populations. Furthermore, the genetic diversity within and among *A. pumilus* populations (particularly in the Mid-Atlantic region) has received little attention from investigators. This observation coupled with the phylogenetic ambiguity of the genus compelled such an analysis.

Presumably, genetic diversity would be high within the morphologically diverse genus *Amaranthus*. Therefore, molecular characters were chosen to test this hypothesis and to limit the bias associated with morphological characters that may be environmentally

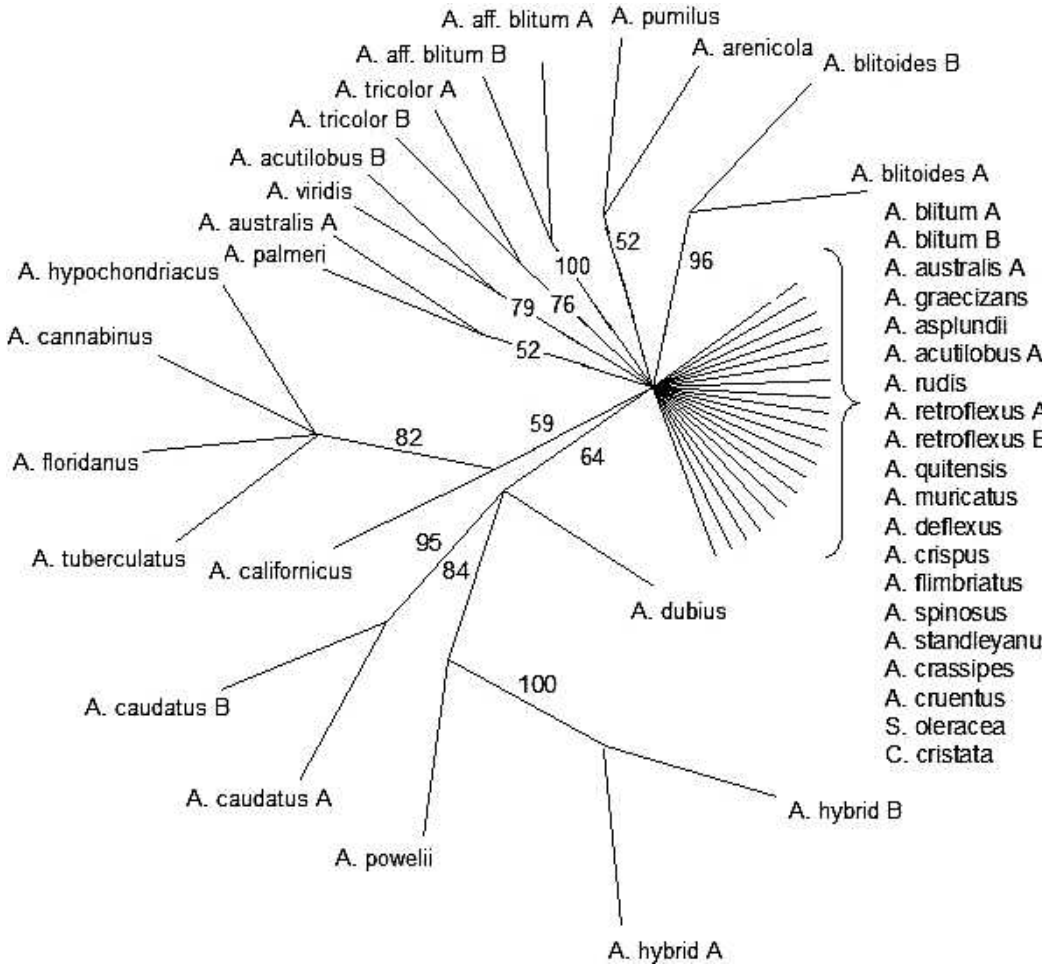

Figure 1. Bayesian consensus tree with posterior probability branch support.

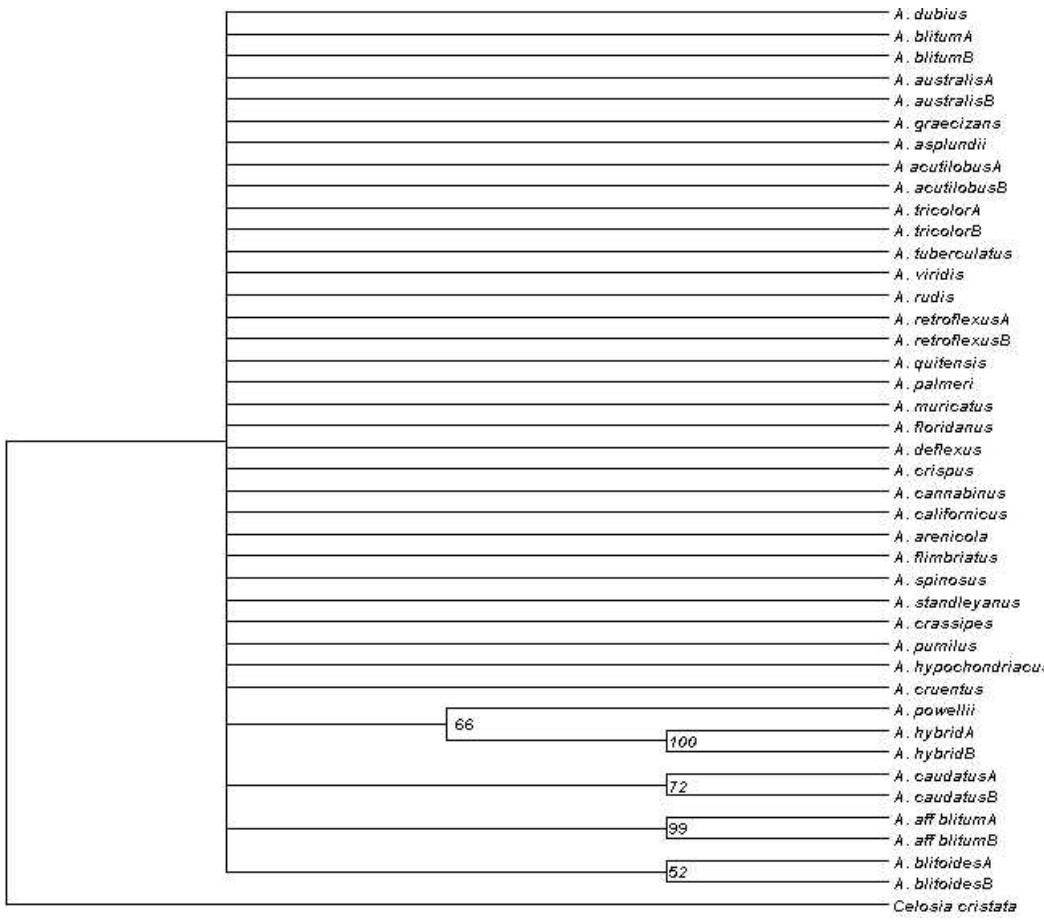

Figure 2. Strict concensus parsimony tree. Statistical branch support is based on 1000 replicates.

influenced. The genetic variation values among *A. pumilus* populations were low while the values for *A. hypochondriacus*, and *A. cruentus* samples are considered relatively high. The difference of these genetic variation values coupled with poorly resolved phylogenetic relationships, suggest that *Amaranthus* may be a recently evolved genus. The propagated specimens on Assateague Island represented individuals from the first, second, and third filial generations, limiting the likelihood for significant genetic variation. Therefore, the low gene diversity values should be interpreted with caution. As these specimens were the result of propagation, they were among the most recent generation available for sampling. Unfortunately, only four individuals were available to represent the wild Assateague population. While the small sample size of the wild Assateague specimens limits inferences about wild population-level genetic diversity, greater variation was still observed when compared to the propagated population. The percentage of polymorphic loci (Table 3) between the wild Assateague specimens and the propagated Assateague specimens was nearly the same despite the larger sampling size of the propagated population. Though genetic variation values for Fenwick Island individuals were higher than the Assateague Island populations, values for all *A. pumilus* (regardless of location) were generally low. The results appear typical of a geographically isolated population arising from founder events, genetic bottlenecks, and suspected infrequent gene flow (Whitaker et al. 2003, Vergeer et al. 2003). Moreover, the reproductive strategy of this selfing species may also contribute to low genetic diversity (Li and Song 2001, Sellars

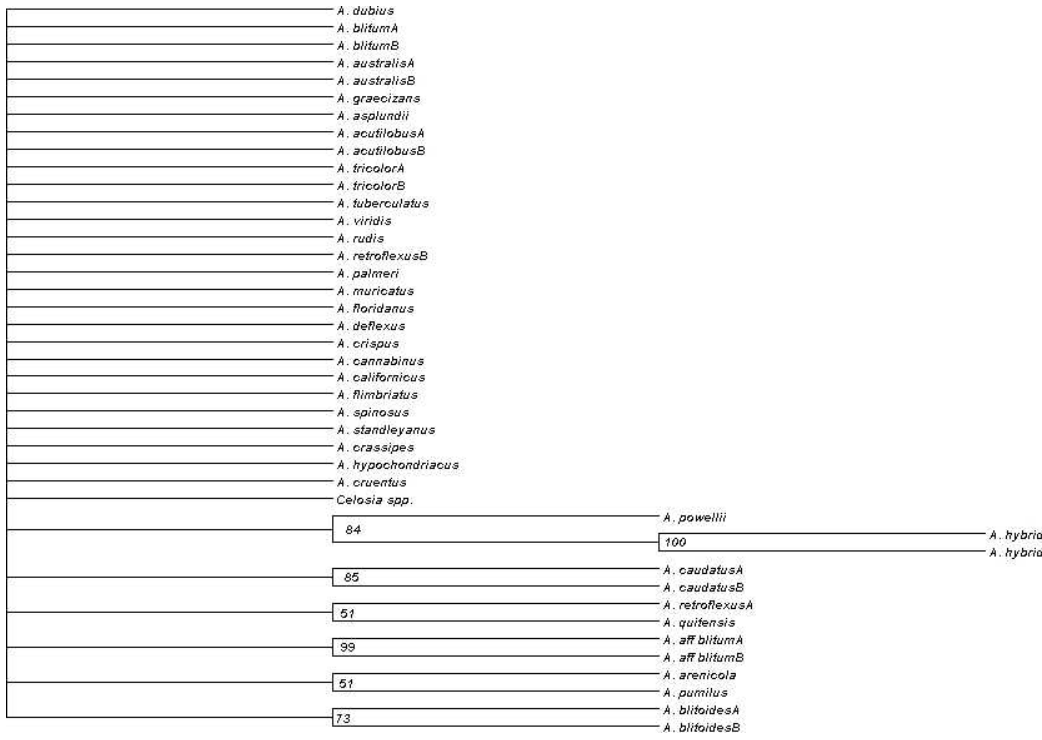

Figure 3. Neighbor-joining phylogenetic tree. Statistical branch support is based on 1,000 replicates.

and Jolls 2007). Haplotypes D, E, and F were unique to the Fenwick population while Haplotypes B was reported in both Assateague and Fenwick populations. Gene flow between populations seems an unlikely scenario given the distance between populations. The continued monitoring of genetic diversity of *A. pumilus* is recommended based on the species susceptibility to founder events and genetic bottlenecks. As the Mid-Atlantic region represents the middle of the species geographic range, Assateague and Fenwick Islands are ideal locations for future studies investigating the level of gene flow among geographically isolated *A. pumilus* populations along the east coast of the United States.

Lack of phylogenetic resolution was common across the variety of tree-construction methods used in this study. The poor resolution of the trees is consistent with Lanoue et al. (1996) who reported similar ambiguous results using phylogenetic analyses based on restriction-site variation in chloroplast and nuclear DNA regions. However, *A. pumilus* consistently paired with *A. arenicola* in both neighboring-joining and Bayesian trees with similar statistical support. This relationship contrasts with former hypotheses that *A. arenicola* is sister to *A. tuberculatus* (Wassom and Tranel 2005). The variation detected between *A. pumilus* and *A. hypochondriacus* supports the conclusions of Marcone (2000) who also discovered much genetic diversity between both species.

Tree length distribution frequency of 10^6 parsimonious trees was analyzed to measure phylogenetic signal. The distribution was left-skewed which may indicate phylogenetic signal within the data matrix. Matrices containing molecular noise produce tree length distribution histograms that are largely symmetrical. Hillis and Huelsenbeck (1992) proposed that tree-length distribution skewness reflects the success of parsimony in determining the ''true phylogeny.'' This was not our finding as a large polytomy was seen in our trees (regardless of method). These close genetic relationships among the majority of the taxa may indicate again that *Amaranthus* is a recently evolved genus. Despite their effectiveness in measuring genetic variation and hypervariability, the

single-primer ISSR technique failed to produce a robust amaranth phylogeny. It is likely that the high level of homoplasy within the data matrix of molecular characters significantly contributed to the poor resolution found across the three tree construction criterion. If so, it is plausible that the phenotypic variation found within Amaranthus is more influenced by environmental factors than by an underlying genetic influence.

SUMMARY This study revealed low levels of genetic variation between isolated wild and propagated *Amaranthus pumilus* populations in the United States Mid-Atlantic region. Our results also support the past conclusions of Marcone (2000), who reported high levels of genetic variation between *A. pumilus* and grain amaranth. ISSR analysis proved to be a useful technique in genetic diversity analyses though they did not provide strongly supported phylogenetic results for the *Amaranthus*. The continued monitoring of genetic diversity of Mid-Atlantic *A. pumilus* populations is recommended.

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