Journal of Heredity 2009:100(3):338–347
doi:10.1093/jhered/esn102
Advance Access publication December 5, 2008

Genetic Diversity of *Amaranthus* Species from the Indo-Gangetic Plains Revealed by RAPD Analysis Leading to the Development of Ecotype-Specific SCAR Marker

Tui Ray and Satyesh Chandra Roy

From the Department of Botany, Centre of Advanced Study, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700019, India. Tui Ray is now at Plant Biology Division, The Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401.

Address correspondence to Satyesh Chandra Roy at the address above, or e-mail: scroy_caluniv@yahoo.com.

Abstract

Genetic diversity and relationships among 6 *Amaranthus* species from 8 phytogeographic regions of the Indo-Gangetic plains were analyzed using a random amplified polymorphic DNA (RAPD) marker. RAPD primers yielded a total of 262 amplicons, ranging from ~250 to ~3000 bp in size with an average of 13.1 amplicons per primer, of which 254 amplicons (96.94%) were polymorphic. The genetic similarity coefficient among all the *Amaranthus* species ranged from 0.16 to 0.97 with a mean similarity coefficient of 0.56, indicating that variation existed in the genetic diversity of different populations. In the unweighted pair group method with arithmetic average dendrogram, populations of the same species clustered together. A unique 1371-bp RAPD band specific for *Amaranthus gangeticus* (syn. *tricolor*) of a particular phytogeographic region was converted to a sequenced characterized amplified region (SCAR) marker. The translated marker sequence showed homology with hemagglutinin protein. This SCAR marker is potentially useful for germplasm conservation and identification of amaranth ecotype.

Key words: *Amaranths, ecotype, RAPD, SCAR*

Amaranths (*Amaranthus* sp.), belonging to the family Amaranthaceae, include the most common leafy vegetables grown in the Indo-Gangetic plains of eastern India. The cultivated amaranths are utilized as food grains, leafy vegetables, and forage crops in diverse geographic areas, such as America, China, Greece, Italy, Russia, Nepal, and India (Stallknecht and Schulz-Schaeffer 1993). The superior nutrition, drought tolerance, disease and pest resistance, high yield in production, and increasing rate of consumption have made this Native American crop more attractive for cultivation in developing countries such as India. Compared with traditional crops, this pseudocereal is rich in protein (17–19% of dry weight) with double the amount of essential amino acids than wheat grain protein (Becker et al. 1981; Bressani et al. 1987). Considering its agronomic importance, attention should be given to the cultivation, conservation, and sustainable utilization of this promising crop.

Despite being a self-pollinated crop, varying amounts of outcrossing and frequent interspecific and intervarietal hybridization have brought wide variation in amaranth genotypes. Amaranths also exhibit tremendous diversity related to their wide adaptability to different ecogeographic situations (Lee et al. 2008). Due to their long cultivation history in various phytogeographic regions of the Indo-Gangetic plains, the local *Amaranthus* species have developed several ecotypes adapted to different environmental conditions, such as cold, drought, and salinity. Exact genotype and/or ecotype identification of an economically important plant is essential for germplasm conservation. However, correct genotype identification in amaranth has been a long-term challenge (Wax 1995). Insights into the relative gene diversity among and within wild populations would be useful in plant breeding and for developing ex situ conservation strategies of plant genetic resources (Bartish et al. 2000). An important topic in conservation studies is the evaluation of genetic isolation (Zhao et al. 2007), and thus, it is necessary to evaluate the genetic diversity of local amaranths for

implementing effective conservation strategies. Identification and preservation of germplasm are necessary for maintaining genetic diversity, studying local genetic material, and even to choose ecotypes having high nutritional interest in their place of origin (Perez-Gonzalez 2001). Considering the huge demand of amaranth genotypes with favorable agronomic traits, a field evaluation was performed to enhance the pool of agronomically useful *Amaranthus* resources (Wu et al. 2000). Although molecular marker-based identification has not been a routine practice, the limited distinctive morphological characters, predominance of ecotypes, and considerable socioeconomic importance of this crop reinforce the need for authenticated identification of species, cultivars, ecotypes, and hybrids using molecular markers.

Molecular markers including the random amplified polymorphic DNA (RAPD) marker have been employed to study the genetic diversity and phylogenetic relationships between *Amaranthus* species (Lee et al. 2008; Wassom and Tranel 2005; Xu and Sun 2001). The RAPD marker has considerable appeal for surveying genomic variation because it is relatively inexpensive, utilizes arbitrary primers, and randomly samples a potentially large number of loci in a less complex pattern than other polymerase chain reaction (PCR)-based markers (Das et al. 2005; Hadrys et al. 1992; Williams et al. 1993). Attempts have been made to employ RAPD analysis for several plant species in relation to development of genetic conservation and improvement strategies (Rao 2004), including the study of genetic diversity and evolutionary relationships between grain amaranths and their putative ancestors (Chan and Sun 1997).

RAPD-based specific and sensitive sequence characterized amplified regions (SCAR) markers have proved to be more reproducible for authenticated identification of genotypes, wherein the RAPD marker termini are sequenced and longer primers (22–24 nucleotides) are designed for specific amplification of particular loci (Paran and Michelmore 1993). The development of species-specific SCAR markers have been reported for several plants, including lettuce (Paran and Michelmore 1993), *Brassica* (Barret et al. 1998), wheat (Hernandez et al. 1999), bamboo (Das et al. 2005), and *Phyllanthus emblica* (Dnyaneshwar et al. 2006).

The aim of this work was to study the genetic diversity and phylogenetic relationships among 6 popular species of *Amaranthus* from different phytogeographic regions of the Indo-Gangetic plains using the RAPD marker. Plants from most of the distribution area of amaranths were examined to develop ecotype-specific SCAR markers based on the polymorphic RAPD fragment. No SCAR marker has previously been generated for the identification of amaranth genotypes. Here, we report development of a potential ecotype-specific SCAR marker for *Amaranthus gangeticus* (syn. *tricolor*).

Materials and Methods

Plant Materials

Plants of 6 *Amaranthus* species, *Amaranthus gangeticus*, *Amaranthus paniculatus*, *Amaranthus viridis*, *Amaranthus hypochondriacus*, *Amaranthus caudatus*, and *Amaranthus cruentus* were collected from 8 phytogeographic regions of the Indo-Gangetic plains: Krishnanagar, Diamond Harbour, Burdwan, Malda, Purulia, Hasnabad, Howrah, and Hasimara (Table 1). Plants were identified by the Botanical Survey of India, Kolkata, India. Details of ecological conditions of the phytogeographic regions are given in Table 1. One population from a phytogeographic region was considered for each *Amaranthus* species; but all 6 species were not available in every region, resulting in a total of 30 populations for the 6 species. Five independent plants per population were sampled randomly for a total of 150 plants.

Isolation of PCR-Compatible Genomic DNA

Genomic DNA was isolated from young leaves of the 150 individual plants using a protocol modified from Dellaporta et al. (1983). Leaves were ground to fine powder and extracted in extraction buffer (50 mM Tris-Cl pH 8.0, 10 mM ethylenediaminetetraacetic acid pH 8.0, 100 mM NaCl, 1% sodium dodecyl sulfate (SDS), and 10 mM 2-mercaptoethanol). The supernatant was extracted twice with phenol:chloroform (1:1) and the DNA was precipitated by centrifugation at 10 000 $\times$ *g* for 10 min after addition of one-tenth volume of 3 M ammonium acetate and equal volume of chilled isopropanol. The pellet was washed with 70% ethanol and suspended in TE buffer. To remove coprecipitated RNA, RNase (Sigma, St Louis, MO) treatment was conducted, followed by polyethylene glycol (PEG 8000) treatment to remove proteins and polysaccharides (Dixit 1998). Quantification and quality verification of total DNA were performed as reported earlier by Ray et al. (2006).

RAPD Analysis

RAPD analyses using 50 decamer primers of Operon kits A, B, D, and E (Operon Technologies, Alameda, CA) followed by gel electrophoresis were done according to the method reported earlier by Ray et al. (2006). Reproducibility of the amplification pattern was checked by repeating each reaction thrice without deliberate alteration in the protocol.

Data Analysis

The bands of equal molecular weight and mobility generated by the same primer were considered to be of identical locus. Only consistently reproducible, well-resolved fragments in the size range of ~250 to ~3000 bp were scored as 1 for the presence and 0 for the absence for each plant. At the intraspecific level, RAPD polymorphism was measured as the proportion of polymorphic loci to the total number of loci scored in all populations of the same species. The similarity matrix and dendrogram were constructed using single-plant data from each population following the NTSYSpc 2.1 (Exeter Software, Setauket, NY) software package (Rohlf 2000). Genetic similarities between

Table 1. Collection sites of *Amaranthus* species from 8 phytogeographic regions of Indo-Gangetic plains with their ecological conditions

Population	Taxon (population code)	Phytogeographic regions	Land forms	Altitude (m)	Latitude	Longitude	Temperature max/min (°C)	Average annual rainfall (cm)	Soil type
I	*Amaranthus gangeticus* (syn. *tricolor*) (*AgI*) *Amaranthus paniculatus* (*ApI*) *Amaranthus viridis* (*AvI*)	Krishnanagar	Ganges delta	14.0	23°24′N	88°33′E	40.1/8.2	140.0	Alluvial
II	*A. gangeticus* (syn. *tricolor*) (*AgII*) *A. paniculatus* (*ApII*) *A. viridis* (*AvII*)	Diamond Harbour	Coastal plain	0.0	22°11′N	88°14′E	35.0/19.0	200.0	Clay loam
III	*A. gangeticus* (syn. *tricolor*) (*AgIII*) *A. paniculatus* (*ApIII*) *A. viridis* (*AvIII*) *Amaranthus hypochondriacus* (*AhIII*) *Amaranthus caudatus* (*AcaIII*)	Burdwan	Rarh region	40.0	23°16′N	87°54′E	36.0/13.0	135.0	Alluvial
IV	*A. gangeticus* (syn. *tricolor*) (*AgIV*) *A. paniculatus* (*ApIV*) *A. viridis* (*AvIV*)	Malda	North Bengal plains	25.0	25°06′N	88°06′E	43.0/7.0	175.0	Alluvial
V	*A. gangeticus* (syn. *tricolor*) (*AgV*) *A. paniculatus* (*ApV*) *A. viridis* (*AvV*) *A. hypochondriacus* (*AhV*) *Amaranthus cruentus* (*AcrV*)	Purulia	Western plateau and high lands	227.0	23°19′N	86°22′E	42.0/5.0	125.0	Laterite
VI	*A. gangeticus* (syn. *tricolor*) (*AgVI*) *A. paniculatus* (*ApVI*) *A. viridis* (*AvVI*)	Hasnabad	Ganges delta	6.0	22°36′N	88°54′E	32.5/19.1	150.0	Deltic alluvial
VII	*A. gangeticus* (syn. *tricolor*) (*AgVII*) *A. paniculatus* (*ApVII*) *A. viridis* (*AvVII*)	Howrah	Ganges delta	12.0	22°35′N	88°20′E	30.7/19.8	160.0	Alluvial
VIII	*A. gangeticus* (syn. *tricolor*) (*AgVIII*) *A. viridis* (*AvVIII*) *A. hypochondriacus* (*AhVIII*) *A. caudatus* (*AcaVIII*) *A. cruentus* (*AcrVIII*)	Hasimara	Terai region	245.0	26°45′N	89°20′E	28.0/4.0	400.0	Alternate layers of clay and sand

Amaranthus gangeticus (syn. *tricolor*), *Amaranthus paniculatus*, and *Amaranthus viridis* are leafy vegetables, and *Amaranthus hypochondriacus*, *Amaranthus caudatus*, and *Amaranthus cruentus* are grain types. Ecological data were provided by Geological Survey of India, India.

populations were measured by the Jaccard's (1908) similarity coefficient with SIMQUAL module. Similarity coefficients were used to construct dendrogram using the unweighted pair group method with arithmetic average (UPGMA) and the sequential, hierarchical, and nested clustering (SHAN) routine in NTSYS program.

Marker Selection

A unique, highly reproducible RAPD band of ~1300 bp, occurring only in population II of *A. gangeticus* (syn. *tricolor*) from Diamond Harbour (*AgII*, Table 1) using primer OPA-20 (5′-GTTGCGATCC-3′), was selected to develop an ecotype-specific SCAR marker and designated as AG1300.

Further confirmation of the putative marker within population II of *A. gangeticus* was done by RAPD analysis using OPA-20 with 40 randomly collected individual plants of *AgII* from Diamond Harbour.

Cloning and Sequencing of Putative RAPD Marker

After RAPD analysis using OPA-20, the ~1300-bp band was gel purified using MinElute gel extraction kit (Qiagen, Valencia, CA). The DNA fragment was cloned in pGEM-T easy vector (Promega, Madison, WI) following the manufacturer's instructions and subsequently transformed into *Escherichia coli* strain DH5α (Sambrook et al. 1989). The size of DNA insert in transformed clones (pGEM-T-AG1300) was screened by PCR analysis with OPA-20 and by *Eco*RI (Roche Diagnostics GmbH, Mannheim, Germany) restriction enzyme digestion. The sequence of AG1300 in pGEM-T-AG1300 was determined bidirectionally using T7 and SP6 promoter–specific primers from Bangalore Genei (Bangalore Genei Pvt Ltd, Bangalore, India) using an automated ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA).

Designing of SCAR Primers and Amplification of Genomic Regions

Based on the sequence of cloned RAPD marker, a specific primer pair was designed ($AGII_{1300}F$ 5′-TCTGTACG-CAACGGGAAACCTGG-3′ and $AGII_{1300}R$ 5′-AACCC-GATGCCGAAAGCTG GAG-3′) using Gene Runner 3.05 software (Hastings software Inc., Hastings, NE) and synthesized by Bangalore Genei. PCR amplification was carried out with 20 pmol of SCAR primer pairs ($AGII_{1300}F$ and $AGII_{1300}R$), 2.5 µl of 10× PCR buffer (MBI Fermentas, Hanover, MD), 2 mM $MgCl_2$, 0.3 mM dNTP mix (MBI Fermentas), 2.5 U *Taq* DNA polymerase (MBI Fermentas), and 1 µg template DNA in 25 µl reaction volume. The amplification cycle consisted of an initial denaturation at 94 °C for 5 min followed by 40 cycles of 60 s at 94 °C, 60 s at 58 °C, and 90 s at 72 °C, and finally terminated with an extension of 5 min at 72 °C. The annealing temperature was adjusted according to the T_m of the primers.

Southern Blot Hybridization

The Southern analysis was carried out according to Sambrook et al. (1989). The genomic DNA of all the plant samples were digested with *Eco*RI (Roche Diagnostics GmbH) and were transferred to positively charged hybond-N^+ nylon membrane (Amersham Biosciences, Buckinghamshire, United Kingdom). For probe preparation, the putative marker AG1300 was digested out from pGEM-T-AG1300 using *Eco*RI (Roche Diagnostics GmbH) and labeled with [α-P^{32}]-dCTP (Perkin Elmer, Walthan, MA) using "Ready Prime" DNA labeling kit (Amersham Biosciences). Hybridization with the probe was carried out overnight at 68 °C followed by washing twice in buffer 1 (2× standard saline citrate [SSC] and 0.1% SDS) at room temperature and twice in buffer 2 (0.1× SSC and 0.1% SDS) at 68 °C for 30 min per wash. Detection was performed after 5 days of exposure to X-ray film at −80 °C.

Analysis of Sequence Data of Putative Marker Using Bioinformatics Tools

The 1371-bp DNA sequence of AG1300 putative SCAR marker from *A. gangeticus* (syn. *tricolor*) of population II from Diamond Harbour was submitted to National Center for Biotechnology Information (NCBI) GenBank (accession number DQ191177). Homology search was done at http://www.ncbi.nlm.nih.gov/BLAST/ of NCBI with the program BlastN and BlastX.

Results and Discussion

Genetic Diversity and Phylogenetic Relationships among *Amaranthus* Species by RAPD Analysis

In the present study, of 50 primers tested, an initial screening taking one plant from each population resulted in selection of 20 decamer primers that produced clear and reproducible RAPD profiles. An analysis of 5 individual plants of 30 populations was conducted with the selected 20 primers. No variation in the RAPD profile was observed within 5 individuals of a species of same population using these 20 primers. To avoid complicacy and redundancy, data from one individual per population were used to construct the dendrogram and study the phylogeny among amaranth populations.

Amplicons in the size range from ~250 to ~3000 bp were scored for an estimation of genetic relationships among *Amaranthus* species. A total of 262 loci were generated, of which 8 were monomorphic, resulting in 96.94% polymorphism at the interspecific level. An average of 13.1 loci per primer was produced, ranging from a minimum of 5 loci using OPB-13 to a maximum of 18 loci using OPA-18. At the interspecific level, 73.8% of bands were polymorphic among the 3 leafy types (*A. gangeticus*, *A. paniculatus*, and *A. viridis*), whereas 71% bands were polymorphic among the 3 grain types (*A. hypochondriacus*, *A. caudatus*, and *A. cruentus*). Although the percentage of polymorphism among leafy amaranths was not much higher than for the grain types in our study, according to Chan and Sun (1997), levels of RAPD polymorphism are higher in leafy amaranths, due to their relatively short cultivation history or lack of selection pressure in domestication. The extent of RAPD polymorphism can be observed among the amaranth populations using OPA-20 (Figure 1).

Due to their tropical origin, leafy amaranths are more commonly found in the Indo-Gangetic plains compared with grain amaranths, which meant that the size of grain amaranth populations were generally smaller in our study. However, a previous report of equal number of plants randomly collected from assorted population sizes showed that trends of genetic diversity do not vary with population size (Zhao et al. 2007).

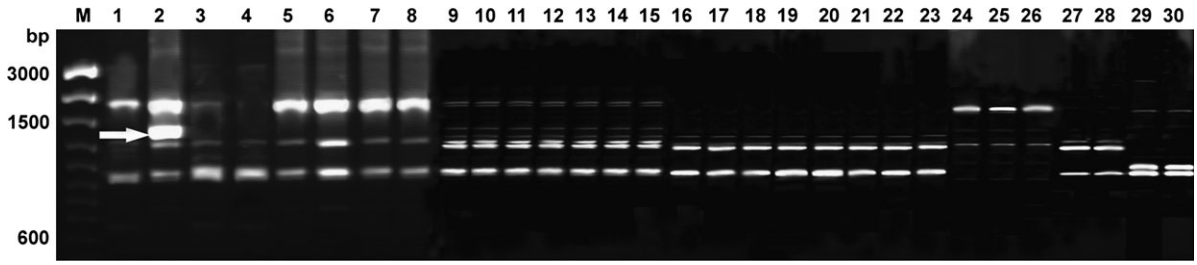

Figure 1. Agarose gel electropherogram of *Amaranthus* species after RAPD analysis with OPA-20. Lane M, 100-bp ladder molecular weight marker; lane 1–8, *Amaranthus gangeticus*; lane 9–15, *Amaranthus paniculatus*; lane 16–23, *Amaranthus viridis*; lane 24–26, *Amaranthus hypochondriacus*; lane 27–28, *Amaranthus caudatus*; lane 29–30, *Amaranthus cruentus* from different populations (I–VIII serially) according to Table 1. The arrow indicates migration of putative 1371-bp band in *A. gangeticus* of population II.

At the intraspecific level, the percentages of RAPD polymorphism were found to be 52.1%, 37.7%, and 39.1% in the leafy amaranths *A. gangeticus*, *A. paniculatus*, and *A. viridis*, respectively, and 22.5%, 18.3%, and 23.3% in the grain types *A. hypochondriacus*, *A. caudatus*, and *A. cruentus*, respectively. Chan and Sun (1997) also reported a higher percentage of polymorphism in leafy amaranths at the intraspecific level. The high percentage of intraspecific polymorphism (52.1%) in *A. gangeticus* indicates that *A. gangeticus* sustains greater genetic variation than the other amaranth species studied. A similarity matrix shows the genetic distance among all the populations of amaranths in this study (Table 2). The dendrogram reflects that in spite of the apparent phenotypic similarity, RAPD markers were able to detect sufficient polymorphisms to distinguish the same plant species collected from different phytogeographic regions (Figure 2). All the populations of a species have clustered together (Figure 2). It is plausible that the primers we used in this study amplified mostly the conserved part of the genome, so they could not show any variation within a population. The RAPD-based UPGMA analysis clearly separated leafy types from grain types into 2 separate clusters (Figure 2), which supports the Sauer's monophyletic hypothesis for the origin of grain and leafy amaranths. In this study, *A. paniculatus* and *A. viridis*, which have distinctly different morphology, showed the highest similarity (Table 2, Figure 2) indicating that they are genetically closest among all the *Amaranthus* species studied. It is also evident from the RAPD-based clustering (Figure 2) that *A. caudatus* is closely related to *A. hypochondriacus*. Similar results were also observed in previous RAPD-based analyses (Chan and Sun 1997; Transue et al. 1994) and hybrid fertility data (Gupta and Gudu 1991). Although Pal and Khoshoo (1973) represented *A. tricolor* as one of the common progenitors of *A. cruentus* and *A. hypochondriacus*, the present study revealed low genetic similarities between *A. gangeticus* (syn. *tricolor*) and other grain amaranths (Table 2), and they were clustered far apart (Figure 2).

According to Sauer (1957) the amount of geographic advances has been very unequal, even for a single species of amaranth along different borders. In some cases, expansion was local, but elsewhere migration is active, causing exceptional hybridization in amaranths (Sauer 1957). Our study showed some geographic cohesiveness, as represented by *ApII* and *ApVII* and *AvII* and *AvVII*, which were collected from close phytogeographic regions and found to be on neighboring branches in the dendrogram (Figure 2). Geographic and ecological differences in the distribution of genetic diversity among amaranth populations were evidenced by other populations of this study. There is always a controversy on the relationship between genetic divergence and geographical origin. Some studies showed little relationship (Hadian et al. 2008), whereas others clearly demonstrated noticeable association between population characteristics and the environment in which they occur (Ge et al. 2003). There is no doubt that when morphological variation causes confusion or misidentification, RAPD analysis can aid the correct identification of species in amaranth genetic resources (Chan and Sun 1997; Transue et al. 1994). However, sensitivity of RAPD reactions to a number of reaction parameters sometimes fails to generate consistent profiles, which necessitates the designing of a more authentic and specific SCAR marker from polymorphic RAPD band (Ellsworth et al. 1993).

Identification and Molecular Analysis of Ecotype-Specific SCAR Marker in *A. gangeticus* (syn. *tricolor*)

A considerable number of primers produced distinct polymorphic profiles among the 6 *Amaranthus* species surveyed. A number of population-specific RAPD bands were noted, but after considering reproducibility and robustness, many potentially population-specific RAPD bands were eliminated from our consideration. The primer OPA-20 consistently amplified a bright, ~1371-bp band in individuals of *AgII* (Figure 1, lane: 2) collected from Diamond Harbour. The same band was absent in the rest of the 7 populations of *A. gangeticus* (syn. *tricolor*) and any other populations of *A. paniculatus*, *A. viridis*, *A. hypochondriacus*, *A. caudatus*, and *A. cruentus* (Figure 1). This specific band occurring in *AgII* was named as AG1300, and further reproducibility of this putative marker was checked by intense analysis of 40 randomly selected individuals of *AgII* collected from the same phytogeographic region (Table 1)

Table 2. Similarity matrix of *Amaranthus* populations based on Jaccard's similarity coefficient

	AgI	*AgII*	*AgIII*	*AgIV*	*AgV*	*AgVI*	*AgVII*	*AgVIII*	*ApI*	*ApII*	*ApIII*	*ApIV*	*ApV*	*ApVI*	*ApVII*	*AvI*	*AvII*	*AvIII*	*AvIV*	*AvV*	*AvVI*	*AvVII*	*AvVIII*	*AhyIII*	*AhyV*	*AhyVIII*	*AcaIII*	*AcaVIII*	*AcrV*	*AcrVIII*
AgI																														
AgII	0.79																													
AgIII	0.84	0.83																												
AgIV	0.88	0.85	0.89																											
AgV	0.83	0.82	0.85	0.87																										
AgVI	0.84	0.83	0.93	0.89	0.85																									
AgVII	0.87	0.86	0.88	0.90	0.88	0.88																								
AgVIII	0.77	0.76	0.78	0.80	0.82	0.78	0.85																							
ApI	0.25	0.26	0.27	0.26	0.26	0.26	0.27	0.26																						
ApII	0.26	0.28	0.29	0.28	0.27	0.28	0.29	0.27	0.89																					
ApIII	0.28	0.29	0.29	0.29	0.28	0.27	0.30	0.27	0.89	0.86																				
ApIV	0.28	0.29	0.30	0.29	0.28	0.28	0.30	0.28	0.93	0.92	0.89																			
ApV	0.28	0.27	0.28	0.27	0.27	0.26	0.31	0.26	0.85	0.83	0.80	0.86																		
ApVI	0.28	0.29	0.30	0.29	0.28	0.28	0.30	0.28	0.92	0.92	0.90	0.94	0.86																	
ApVII	0.29	0.30	0.31	0.30	0.29	0.29	0.31	0.29	0.87	0.89	0.84	0.90	0.83	0.90																
AvI	0.28	0.29	0.29	0.30	0.29	0.27	0.30	0.28	0.71	0.71	0.73	0.72	0.65	0.72	0.69															
AvII	0.29	0.30	0.30	0.31	0.29	0.28	0.31	0.29	0.69	0.66	0.75	0.69	0.64	0.69	0.66	0.86														
AvIII	0.25	0.25	0.26	0.26	0.26	0.24	0.27	0.27	0.70	0.68	0.70	0.68	0.64	0.69	0.68	0.88	0.83													
AvIV	0.27	0.28	0.29	0.29	0.27	0.27	0.29	0.27	0.74	0.73	0.76	0.74	0.69	0.75	0.72	0.93	0.90	0.90												
AvV	0.26	0.27	0.27	0.27	0.25	0.25	0.27	0.25	0.71	0.69	0.73	0.72	0.68	0.72	0.72	0.89	0.84	0.86	0.91											
AvVI	0.27	0.28	0.29	0.29	0.27	0.27	0.29	0.27	0.73	0.71	0.75	0.72	0.67	0.74	0.71	0.93	0.88	0.92	0.96	0.91										
AvVII	0.28	0.28	0.29	0.30	0.27	0.28	0.30	0.29	0.70	0.69	0.71	0.68	0.65	0.72	0.69	0.87	0.86	0.84	0.91	0.85	0.91									
AvVIII	0.26	0.28	0.28	0.28	0.26	0.26	0.29	0.27	0.72	0.70	0.74	0.71	0.66	0.73	0.70	0.90	0.87	0.87	0.97	0.88	0.92	0.88								
AhyIII	0.16	0.16	0.17	0.17	0.16	0.16	0.17	0.16	0.15	0.16	0.16	0.16	0.15	0.16	0.17	0.15	0.15	0.15	0.16	0.14	0.16	0.15	0.16							
AhyV	0.14	0.16	0.17	0.17	0.16	0.17	0.17	0.16	0.15	0.16	0.15	0.15	0.15	0.15	0.16	0.15	0.15	0.15	0.16	0.14	0.16	0.16	0.16	0.87						
AhyVIII	0.14	0.14	0.15	0.15	0.15	0.14	0.15	0.14	0.13	0.13	0.13	0.14	0.15	0.15	0.14	0.12	0.12	0.13	0.13	0.13	0.13	0.12	0.13	0.88	0.81					
AcaIII	0.20	0.20	0.21	0.22	0.23	0.22	0.22	0.21	0.16	0.17	0.14	0.16	0.17	0.16	0.18	0.14	0.15	0.15	0.14	0.13	0.15	0.14	0.15	0.39	0.40	0.38				
AcaVIII	0.19	0.18	0.20	0.20	0.21	0.21	0.21	0.19	0.14	0.14	0.12	0.13	0.15	0.13	0.15	0.12	0.13	0.13	0.12	0.11	0.13	0.13	0.13	0.41	0.42	0.42	0.82			
AcrV	0.18	0.19	0.20	0.20	0.19	0.18	0.20	0.19	0.17	0.20	0.18	0.19	0.19	0.19	0.20	0.19	0.17	0.18	0.19	0.18	0.19	0.18	0.19	0.38	0.34	0.38	0.40	0.40		
AcrVIII	0.16	0.18	0.18	0.18	0.17	0.17	0.18	0.18	0.14	0.17	0.16	0.16	0.16	0.18	0.17	0.16	0.16	0.16	0.17	0.16	0.17	0.16	0.17	0.36	0.32	0.38	0.35	0.33	0.77	

Names are abbreviated according to population code of Table 1.

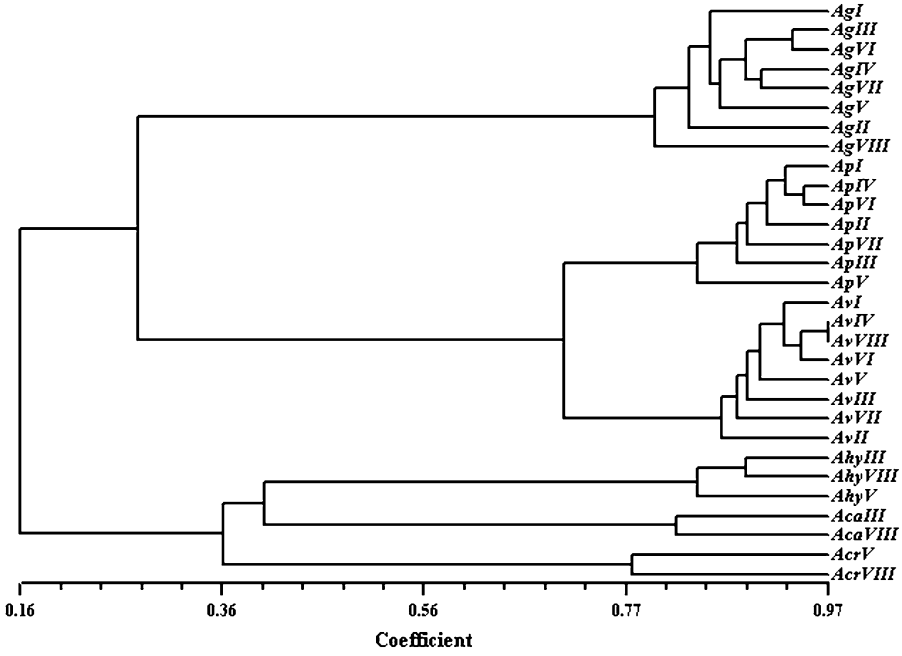

Figure 2. UPGMA-based dendrogram derived from RAPD analyses of *Amaranthus* species and their populations. Names abbreviated according to Table 1.

with unaltered RAPD reaction using OPA-20 (Figure 3). Consistent amplification of the same band AG1300 in all 40 individuals of *AgII* without any exception moved us to select and sequence this highly reproducible putative marker. The 1371-bp DNA sequence (Figure 4) was submitted to NCBI GenBank under accession number DQ191177. The sequence showed the presence of nucleotide sequences of OPA-20 on both ends with 47.92% G + C content without any repeats (Figure 4).

In order to make the ecotype selection trouble free as well as more accurate, designing a SCAR primer from a putative RAPD band for amplification of a specific locus becomes essential. In our study, it was achieved by designing a specific SCAR primer pair within the 1371-bp amplifiable sequence of AG1300 as shown in Figure 4. PCR amplification of genomic DNA from 8 populations of *A. gangeticus* (syn. *tricolor*) and other populations of *A. paniculatus*, *A. viridis*, *A. hypochondriacus*, *A. caudatus*, and *A. cruentus* showed a single, distinct, brightly resolved ~1250-bp band only in *AgII* (Figure 5). No such amplification was observed in *A. gangeticus* of the other 7 populations or other species of *Amaranthus* of this study (Figure 5). Presence of a single ~1250-bp expected band in the PCR product from genomic DNA of 40 individuals of *AgII* further confirmed the occurrence of the SCAR marker within *A. gangeticus* of Diamond Harbour (Figure 6). Specific marker development for any plant taxon requires consistently reproducible genomic sequence, especially when they are used for germplasm selection. This SCAR marker can be used consistently for identifying *A. gangeticus* from the coastal region of Diamond Harbour.

Paran and Michelmore (1993) reported that RAPD products are not suitable for using as hybridization probes because they often contain repetitive DNA sequences. Absence of any repetitive sequence in AG1300 made this

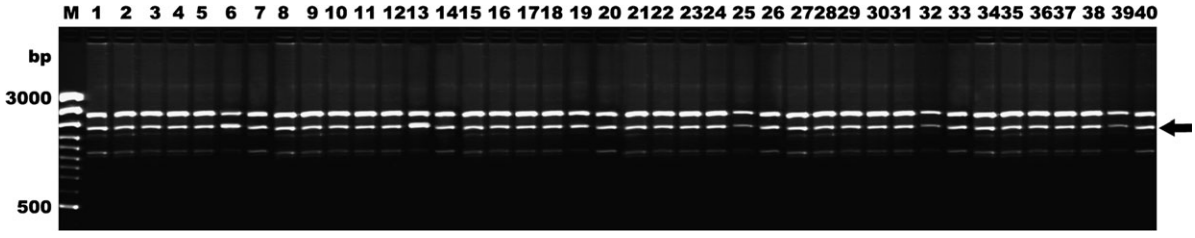

Figure 3. Agarose gel electropherogram of randomly selected individuals of *Amaranthus gangeticus* of population II after RAPD analysis with OPA-20. Lane M, 100-bp ladder molecular weight marker; lane 1–40, 40 individual plants of *A. gangeticus* from population II. The arrow indicates uniform migration of putative 1371-bp band (AG1300).

```
1    GTTGCGATCCGCGGTGCTGACTAGCAGTGTTGATCAATATCGAATGCGTCTGTACGCAACGGGAAACCTGGT
       OPA-20                                          AGII1300F
73   TATTACCAAGATGAGTGGTGGTGTGGAAACATCAGAACCACTTGCTATTTCCGAAGCCAGTTCTTTTGGCGTC
146  GGGGATGTGGTTGTCTTCCGTAGAATGATGAACCCGGAAAAGACCAAAGCATATATCACCGTCAGAGTAAAT
218  GATGTTGAGGTTGCTGCGTGGATTGATGAAACGCCACTGGCTGCGGGTGGTTTCGTGGCGTTTACATCGGGT
290  TCGAGGACGGTTACGTATGATTATTGGATCATTCGAACCATGAATGCTCCTACTACGGCTAACGAACCAGTTC
363  TTGCAGCTGCTAATTTGGTCATGACCAAAAACTCTCCGGTGGATTTCCAGTTTACAGCTTCCGGCGATCCGAT
436  TATTACGTATTCGGTTCGTAGTGGCCTTCCGGACGGTCTGTCTCTGTCTACTACTGGTAATCTTACTGGTATTC
510  CTACCGTGTCGGGTGCTCAAACTATGGTTATTCGAGCAACTAACAGTGTGGGTTATAACGATCGTACATATAC
581  ATTTACGATTGCTGAGCCTGGCACTACCGGTGGGGGTAGCACTGGAGGAGGTTCTACGGCGGCTGGTGTGT
654  ATGGATCCAACGGGACTTATTGGCCTGCGTTTACACCTCGATTGAGCGACTCGTTTGATAAAGTATACACCGT
727  GGCGCCTTCGTGGACTGCGGTTGCTAATGCATTTAAAGATGCCCTAGCCACGACAGCATTGACTGCCAAAGT
799  CGTTATTTATGTCACTCCTGGTACGTTTTCCGCTGGCTCTGGCGCCGGGTCTTCGAACCCTGGAGTTATGCAG
872  AATATCGGAACAACGGGCCGTAAGTGGAATATTGTTGTCACGCCTCGTGACGCTTTGGGTACGGTTAAAGTAT
945  CACCGACGCATGGATCGCCATTTGTCAACCTAAGTGGTATTTCTTTGGTTGGTATTGATTTCACAGGCGCTGC
1018 GGTGATGATGCGAAATTGTAATCGTATGTATATTTCACGTACGTTTGCTCGGCAGGGTAAATTTACAGCCAAC
1091 GGATCTCTTGGTCTTAACAACTGTGAATATGTGGAATGCGTGTTTGGTCCGGAAATTTATGTTTCTGATATTGA
1165 CCGAGCAGACATGCGTATGGAAGATGGCTTTGCGTCTAACAACAGTGGTTTCCGCGGCTGTTATTTCGGACC
1237 TAGTTATAAGATGGATGGTCAAACAGGACATTGTGACACCCTCCAGCTTTCGGCATCGGGTTCTTCCGCCAAT
                                          AGII1300R
1310 ATCAAAGCGTTCTTGTCGGTTTGTGTGTTGTTTGCTTCTTCTAATCAAGGATGGATCGCAAC
                                                       OPA-20
```

Figure 4. Nucleotide sequence of RAPD band AG1300 (GenBank accession number DQ191177). The sequence of OPA-20 and nucleotide sequence used to design SCAR primers ($AGII_{1300}F$ and $AGII_{1300}R$) are underlined.

marker problem free for using as a hybridization probe. In the present study, we used Southern blot analysis to establish the occurrence of homologous sequence (AG1300) in the genome of *AgII*. It was observed in some cases that the RAPD gel could not reveal homologous sequences of different molecular weight, whereas hybridization results recognized those same sequences with a RAPD derived probe (Das et al. 2005; Xu et al. 1995). The strong single hybridization signal as shown in Figure 7 authenticated the unique presence of AG1300 sequence in the genome of *AgII* and proved this marker development successful. Absence of any nonspecific band confirmed the absence of any homologous sequence in other populations of *A. gangeticus* collected from different phytogeographic regions or in any *Amaranthus* of this study. It even eliminated the possibility of the presence of any homologous or duplicate sequence in the genome of *AgII*.

Figure 5. PCR amplification of genomic DNA from *Amaranthus* species using $AGII_{1300}F$ and $AGII_{1300}R$ SCAR primer pairs. Lane M, 100-bp ladder molecular weight marker; lane 1–8, *Amaranthus gangeticus* from populations 1–8; lane 9–10, *Amaranthus paniculatus* and *Amaranthus viridis* from population II; lane 11–13, *Amaranthus hypochondriacus*, *Amaranthus caudatus*, and *Amaranthus cruentus* from population VIII, respectively.

Sequence Data Analysis and Characterization of Ecotype-Specific SCAR Marker

It has always been stated that without DNA hybridization or sequence data analysis, it is unjustified to establish that RAPD bands amplified in individuals of undefined relationships are homologous (Weeden et al. 1992). The Southern blot analysis already affirmed the uniqueness of the ecotype-specific marker (AG1300) in the genome of *AgII*. However, the basic local alignment search tool (BLAST) search of AG1300 sequence (Figure 4) with the NCBI nucleotide database using BlastN tool did not show any significant match with any known nucleotide sequence, which confirmed the first report of this SCAR marker. On the other hand, the BlastX tool result showed a significant match with a putative hemagglutinin related protein (accession number NP 518236; score bit 56.2, *E* value = 5×10^{-06}) (Salanoubat et al. 2002). Occurrence of hemagglutinins in plants like *Amaranthus leucocarpus* (Calderon de la Barca et al. 1985) and *A. caudatus* (Wu et al. 2006) was also reported earlier. It has been advocated that natural selection is the principal force shaping the genetic architecture in natural plant populations (Linhart and Grant 1996). Moreover, there are many reports of evolutionary adaptation in response to environmental changes (Lee 2002), which supports the view that long-term cultivation of *A. gangeticus* in a particular ecological condition of a specific

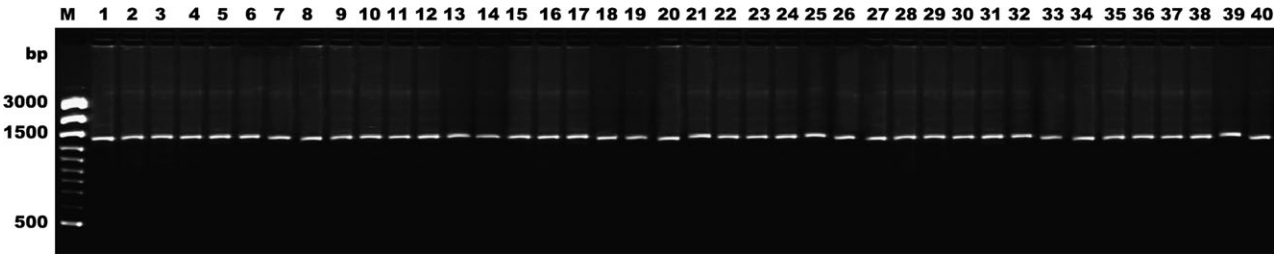

Figure 6. PCR amplification of genomic DNA from 40 individuals of *Amaranthus gangeticus* from population II using $AGII_{1300}F$ and $AGII_{1300}R$ SCAR primer pairs. Lane M, 100-bp ladder molecular weight marker; lane 1–40, 40 individual plants of *A. gangeticus* from population II used in the RAPD analysis. All the lanes showed amplification of 1250-bp band.

region (Diamond Harbour region) might have brought this stable genetic change.

Summary

The present RAPD analysis clearly revealed genetic diversity among and within *Amaranthus* species of Indo-Gangetic plains. The study of genetic diversity among populations of different phytogeographic regions is important, as survival, perpetuation, and continuance of a species to meet the demands of changing environments largely depend on the extent of variability available in its gene pool. Populations of the present study did not show any interspecific hybridization and maintained taxonomic distinctness, as shown by the grouping within their species in the dendrogram. Designing SCAR primers from polymorphic RAPD bands is helpful for plants like amaranths where accurate identification of taxonomic status persists as a long-term problem. This report of developing an ecotype-specific SCAR marker will help to identify the ecotype of *A. gangeticus* (syn. *tricolor*) and to find strategies for marker-assisted identification of amaranth genotypes and conservation of amaranth genetic resources.

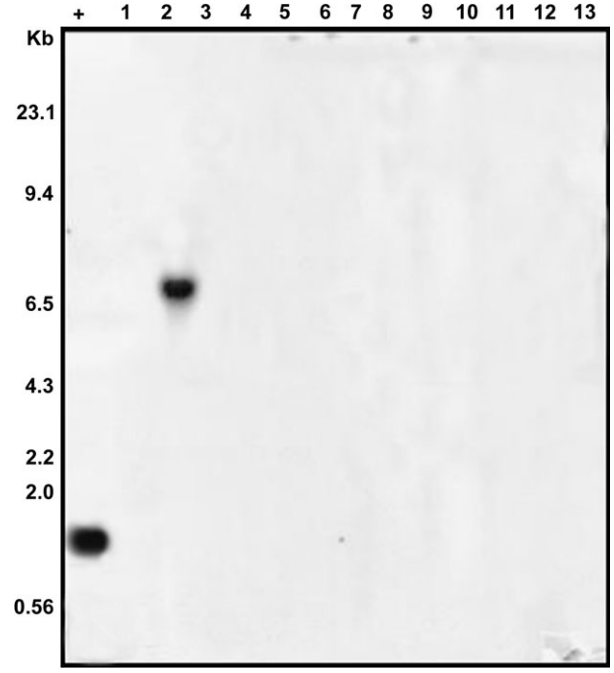

Figure 7. Southern blot analysis for the presence of AG1300 sequence within the genome of *Amaranthus gangeticus* of population II. Genomic DNA was digested with *Eco*RI restriction enzyme and processed for Southern hybridization. Lane +, *Eco*RI digested 1250-bp fragment from pGEM-T-AG1300 as positive control; lane 1–8, *A. gangeticus* from populations 1–8; lane 9–10, *Amaranthus paniculatus* and *Amaranthus viridis* from population II; lane 11–13, *Amaranthus hypochondriacus*, *Amaranthus caudatus*, and *Amaranthus cruentus* from population VIII, respectively. Lane 2 showed single strong hybridizable band in *A. gangeticus* of population II.

Funding

Department of Biotechnology, Government of India, India (D.O. BT/PR/1944/AGR/08/142/2000).

References

Barret P, Delourme R, Foisset N, Renard M. 1998. Development of a SCAR (sequence characterized amplified region) marker for molecular tagging of the dwarf BREIZH (Bzh) gene in *Brassica napas* L. Theor Appl Genet. 97:828–833.

Bartish IV, Garkava LP, Rumpunen K, Nybom H. 2000. Phylogenetic relationships and differentiation among and within populations of *Chaenomeles* Lindl. (Rosaceae) estimated with RAPDs and isozymes. Theor Appl Genet. 101:554–563.

Becker R, Wheeler EL, Lorenz K, Stafford AE, Grosjean OK, Betschart AA, Saunders RM. 1981. A compositional study of amaranth grain. J Food Sci. 46:1175–1180.

Bressani R, Gonzales JM, Zuniga J, Brauner M, Elias LG. 1987. Yield, selected chemical composition and nutritive value of 14 selections of amaranth grain representing four species. J Sci Food Agric. 38:347–356.

Calderon de la Barca AM, Ochoa JL, Valencia ME. 1985. Effects of the extraction of a hemagglutinin on the nutritive value of *Amaranthus leucocarpus* seeds. J Food Sci. 50:1700–1702.

Chan KF, Sun M. 1997. Genetic diversity and relationships detected by isozyme and RAPD analysis of crop and wild species of *Amaranthus*. Theor Appl Genet. 95:865–873.

Das M, Bhattacharya S, Pal A. 2005. Generation and characterization of SCARs by cloning and sequencing of RAPD products: a strategy for species-specific marker development in bamboo. Ann Bot. 95:835–841.

Dellaporta SL, Wood J, Hicks JB. 1983. A plant DNA mini-preparation: version II. Plant Mol Biol Rep. 1:19–21.

Dixit A. 1998. A simple and rapid procedure for isolation of *Amaranthus* DNA suitable for fingerprint analysis. Plant Mol Biol Rep. 16:1–8.

Dnyaneshwar W, Chavan P, Joshi K, Patwardhan B. 2006. Development and application of RAPD-SCAR marker for identification of *Phyllanthus emblica* Linn. Biol Pharm Bull. 29:2313–2316.

Ellsworth DL, Rittenhouse KD, Honeycutt RL. 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. Biotechniques. 14:214–217.

Ge XJ, Yu Y, Zhao NX, Chen HS, Qi WQ. 2003. Genetic variation in the endangered Inner Mongolia endemic shrub *Tetraena mongolica* Maxim (Zygophyllaceae). Biol Conserv. 111:427–434.

Gupta VK, Gudu S. 1991. Interspecific hybrids and possible phylogenetic relations in grain amaranths. Euphytica. 52:33–38.

Hadian J, Tabatabaei SMF, Naghavi MR, Jamzad Z, Ramak-Masoumi T. 2008. Genetic diversity of Iranian accessions of *Satureja hortensis* L. based on horticultural traits and RAPD markers. Sci Hortic. 115:196–202.

Hadrys H, Balick M, Schierwater B. 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol Ecol. 1:53–63.

Hernandez P, Martin A, Dorado G. 1999. Development of SCARs by direct sequencing of RAPD products a practical tool for the introgression and marker assisted selection of wheat. Mol Breed. 5:245–253.

Jaccard P. 1908. Nouvelles recherches sur la distribution florale. Bull Soc Vaud Sci Nat. 44:223–270.

Lee CE. 2002. Evolutionary genetics of invasive species. Trends Ecol Evol. 17:386–391.

Lee JR, Hong GY, Dixit A, Chung JW, Ma KH, Lee JH, Kang HK, Cho YH, Gwag JG, Park YJ. 2008. Characterization of microsatellite loci developed for *Amaranthus hypochondriacus* and their cross-amplification in wild species. Conserv Genet. 9:243–246.

Linhart YB, Grant MC. 1996. Evolutionary significance of local genetic differentiation in plants. Annu Rev Ecol Syst. 27:237–277.

Pal M, Khoshoo TN. 1973. Evolution and improvement of cultivated amaranths. VI. cytogenetic relationships in grain types. Theor Appl Genet. 43:242–251.

Paran I, Michelmore RW. 1993. Development of reliable PCR based markers linked to downy mildew resistance genes in lettuce. Theor Appl Genet. 85:985–993.

Perez-Gonzalez S. 2001. The importance of germplasm preservation and use for temperate zone fruit production in the tropics and subtropics. In: Perez-Gonzalez S, Dennis F, Mondragon C, Byrne D, editors. VI International symposium on temperate fruit growing in the tropics and subtropics. Mexico: Acta Hort (ISHS). 565: p. 25–32.

Ray T, Dutta I, Saha P, Das S, Roy SC. 2006. Genetic stability of three economically important micropropagated banana (*Musa* spp.) cultivars of lower Indo-Gangetic plains, as assessed by RAPD and ISSR markers. Plant Cell Tissue Organ Cult. 85:11–21.

Rao NK. 2004. Plant genetic resources: advancing conservation and use through biotechnology. Afr J Biotechnol. 3:136–145.

Rohlf FJ. 2000. NTSYS-pc: numerical taxonomy and multivariate analysis system. Setauket (NY): Exeter Software.

Salanoubat M, Genin S, Artiguenave F, Gouzy J, Mangenot S, Arlat M, Billault A, Brottier P, Camus JC, Cattolico L, et al. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature. 415: 497–502.

Sambrook J, Maniatis T, Fritsch EF. 1989. Molecular cloning: a laboratory manual. 1v, 2v. Cold Spring Harbor (NY): Cold Springer harbor laboratory Press. p. 1.116–1.118, 6.39–6.58.

Sauer J. 1957. Recent migration and evolution of the dioecious amaranths. Evolution. 11:11–31.

Stallknecht GF, Schulz-Schaeffer JR. 1993. Amaranth rediscovered. In: Janick J, Simon JE, editors. New crops. New York: Wiley. p. 211–218.

Transue DK, Fairbanks DJ, Robison LR, Andersen WR. 1994. Species identification by RAPD analysis of grain amaranth genetic resources. Crop Sci. 34:1385–1389.

Wassom JJ, Tranel PJ. 2005. Amplified fragment length polymorphism-based genetic relationships among weedy *Amaranthus* species. J Hered. 96:410–416.

Wax LM. 1995. Pigweeds of the Midwest—distribution, importance, and management. Proc Iowa Integr Crop Manag Conf. 7:239–242.

Weeden NF, Timmerman GM, Hemmat M, Kneen BE, Lodhi MA. 1992. Inheritance and reliability of RAPD markers. In: Neff M, editor. Joint plant breeding symposia series: application of RAPD technology to plant breeding. Madison (WI): Crop Science Society of America. p. 12–17.

Williams JGK, Hanafey MK, Rafalski JA, Tingey SV. 1993. Genetic analysis using random amplified polymorphic DNA markers. Methods Enzymol. 218:704–740.

Wu H, Sun M, Yue S, Sun H, Cai Y, Huang R, Brenner D, Corke H. 2000. Field evaluation of an *Amaranthus* genetic resource collection in China. Genet Resour Crop Evol. 47:43–53.

Wu J, Luo X, Guo H, Xiao J, Tian Y. 2006. Transgenic cotton, expressing *Amaranthus caudatus* agglutinin, confers enhanced resistance to aphids. Plant Breed. 125:390–394.

Xu F, Sun M. 2001. Comparative analysis of phylogenetic relationships of grain amaranths and their wild relatives (*Amaranthus*; Amaranthaceae) using internal transcribed spacer, amplified fragment length polymorphism, and double-primer fluorescent intersimple sequence repeat markers. Mol Phylogenet Evol. 21:372–387.

Xu H, Wilson DJ, Arulsekar S, Bakalinsky AT. 1995. Sequence specific polymerase chain reaction markers derived from amplified polymorphic DNA markers for fingerprinting grape (*Vitis*) rootstocks. J Am Soc Hortic Sci. 120:714–720.

Zhao W, Wang Y, Chen T, Jia G, Wang X, Qi J, Pang Y, Wang S, Li Z, Huang Y, et al. 2007. Genetic structure of mulberry from different ecotypes revealed by ISSRs in China: an implications for conservations local mulberry varieties. Sci Hortic. 115:47–55.

Received April 2, 2008; Revised November 2, 2008; Accepted November 10, 2008

Corresponding Editor: Perry Gustafson