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Analysis of the Genetic Diversity and Population Structure of Amaranth Accessions from South America Using 14 SSR Markers

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ABSTRACT Amaranth (*Amaranthus* sp. L.) is an important group of plants that includes grain, vegetable, and ornamental types. Centers of diversity for Amaranths are Central and South America, India, and South East Asia, with secondary centers of diversity in West and East Africa. The present study was performed to determine the genetic diversity and population structure of 75 amaranth accessions: 65 from South America and 10 from South Asia as controls using 14 SSR markers. Ninety-nine alleles were detected at an average of seven alleles per SSR locus. Model-based structure analysis revealed the presence of two subpopulations and 3 admixtures, which was consistent with clustering based on the genetic distance. The average major allele frequency and polymorphic information content (PIC) were 0.42 and 0.39, respectively. According to the model-based structure analysis based on genetic distance, 75 accessions (96%) were classified into two clusters, and only three accessions (4%) were admixtures. Cluster 1 had a higher allele number and PIC values than Cluster 2. Model-based structure analysis revealed the presence of two subpopulations and three admixtures in the 75 accessions. The results of this study provide effective information for future germplasm conservation and improvement programs in *Amaranthus*.

Keywords : Amaranth, genetic diversity, population structure, SSRs

Amaranthaceae, to which *Amaranthus* sp. L. belong, includes approximately 87 species, 40 of which are considered native to the Americas. These species can be broadly categorized into grains, green leaf vegetables, and weed types, including pigweeds (Mujica and Jacobsen, 2003), which have a worldwide distribution (Xu and Sun, 2001; Costea *et al*., 2004). Hybrids of amaranth are widely cultivated as ornamental, pseudo-cereal and fodder crops in many tropical to warm-temperate regions of the world (Sauer, 1950, 1967). Most cultivated amaranths are used for various purposes, such as food grains, leafy vegetables, and forage crops in diverse geographic areas, such as the Americas, China, Greece, Italy, Russia, Nepal, and India (Stallknecht and Schulz-Schaeffer, 1993).

The amaranth grain is a valuable food as it has a high protein content and well-balanced amino acid profile (Gamel *et al*., 2006). Analyses of the chemical composition and nutritional value of amaranth grain (Bressani *et al*., 1987; Dodok *et al*., 1997; Andrasofszky *et al*., 1998) confirmed its potential for use in human and animal nutrition as well as medicine (Oke, 1983; Teutenico and Knorr, 1985). With the increasing need to explore alternate sources of food, it is necessary to accelerate and expand the production of amaranths. Amaranths have high photosynthetic efficiency, low input requirements, high yield potential for grain, vegetable, and fodder production, and relatively higher tolerance to biotic and abiotic stresses, such as drought, diseases, and pests (Tucker, 1986).

Most species of amaranths are easily crossbred, and even weedy types will cross with the intended crop if not rogued from the field (Brien and Price, 2008). Taxonomic classification is difficult, as it is necessary to consider characteristics such as pigmentation that shows a wide degree of variation as well as size of the plant, which depends on the number of hours of sunlight and other environmental variables. Finally, the amaranth plant has high plasticity (Espitia, 1986). Information on the genetic diversity and relationships within and among crop species and their wild relatives is essential for efficient utilization of plant germplasm. In addition, they also exhibit marked

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morphological diversity and adaptability to a range of eco-geographical conditions. Due to the agro-economic importance of amaranths, several studies of isozymes and various DNA markers have been performed to understand intra- and interspecific genetic diversity and/or evolutionary relationships (Lanoue *et al*., 1996; Chan and Sun, 1997; Sun *et al*., 1999).

Several valuable methods for molecular marker systems, such as restriction-fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), sequence-tagged sites (STS), amplified fragment length polymorphisms (AFLP), simple sequence repeats (SSRs) or microsatellites, and single nucleotide polymorphisms (SNPs), have been developed and applied for estimation of genetic diversity (Suh *et al*., 1997; Nagaraju et al., 2002; Feltus *et al*., 2004; Bao et al., 2006; Zhao et al., 2009; Li *et al*. 2012). Various marker systems have been used specifically to investigate genetic diversity (Tam *et al*., 2005). Microsatellites or simple sequence repeats (SSRs) have been shown to be valuable for genetic studies (Lewers *et al*., 2005; Moe *et al*., 2010) and can be used to identify genetic relationships among closely related species or genera for molecular ecology and evolutionary biology, so as to address the mechanism(s) involved in population divergence and speciation (Barbara *et al*., 2007) or for studying genome evolution (Rousseau-Gueutin *et al*., 2008).

The objective of this study was to evaluate the genetic diversity and population structure of 75 accessions of amaranths collected from 12 countries, mainly from South America, conserved in the National Genebank of the Rural Development Administration, Republic of Korea (RDA-Genebank), using 14 selected polymorphic SSR markers.

MATERIALS AND METHODS

Plant Materials

A total of 75 amaranth accessions collected from 12 countries (65 from South America for diversity and 10

Table 1. List of the 75 accessions and their model-based clusters. (continued)

No.[1)]	Species	Acc. No.[2)]	Inferred Cluster[3)]	Country of Origin	Provided Country
1	*Amaranthus* sp.	44	1	THA	THA
2	*Amaranthus blitum*	73	1	BRA	USA
3	*Amaranthus blitum*	74	1	PRT	USA
4	*Amaranthus caudatus*	85	1	BOL	USA
5	*Amaranthus caudatus*	89	1	PER	USA
6	*Amaranthus caudatus*	90	1	PER	USA
7	*Amaranthus caudatus*	91	1	PER	USA
8	*Amaranthus caudatus*	92	1	PER	USA
9	*Amaranthus caudatus*	93	1	PER	USA
10	*Amaranthus caudatus*	94	1	PER	USA
11	*Amaranthus caudatus*	95	1	BOL	USA
12	*Amaranthus caudatus*	98	1	NPL	USA
13	*Amaranthus caudatus*	101	1	PER	USA
14	*Amaranthus cruentus*	114	2	MEX	USA
15	*Amaranthus deflexus*	115	1	ARG	USA
16	*Amaranthus deflexus*	116	1	PRT	USA
17	*Amaranthus dubius*	125	Admx	NPL	USA
18	*Amaranthus fimbriatus*	127	2	MEX	USA
19	*Amaranthus graecizans* ssp. *silvestris*	130	1	PRT	USA
20	*Amaranthus hybridus*	137	Admx	PER	USA

Table 1. List of the 75 accessions and their model-based clusters. (continued)

No.[1)]	Species	Acc. No.[2)]	Inferred Cluster[3)]	Country of Origin	Provided Country
21	*Amaranthus hybridus*	138	1	ECU	USA
22	*Amaranthus hybridus*	139	1	ECU	USA
23	*Amaranthus hypochondriacus*	143	2	PRI	USA
24	*Amaranthus hypochondriacus*	147	2	MEX	USA
25	*Amaranthus hypochondriacus*	148	2	MEX	USA
26	*Amaranthus hypochondriacus*	151	2	ARG	USA
27	*Amaranthus palmeri*	170	2	MEX	USA
28	*Amaranthus powellii*	171	2	PRT	USA
29	*Amaranthus quitensis*	186	1	BRA	USA
30	*Amaranthus quitensis*	187	1	PER	USA
31	*Amaranthus quitensis*	188	Admx	PER	USA
32	*Amaranthus quitensis*	190	1	ECU	USA
33	*Amaranthus quitensis*	191	1	BOL	USA
34	*Amaranthus quitensis*	192	1	ECU	USA
35	*Amaranthus quitensis*	193	1	PER	USA
36	*Amaranthus quitensis*	194	1	BOL	USA
37	*Amaranthus spinosus*	213	2	IDN	USA
38	*Amaranthus spinosus*	214	2	THA	USA
39	*Amaranthus standleyanus*	215	1	ARG	USA
40	*Amaranthus standleyanus*	216	1	ARG	USA
41	*Amaranthus tricolor*	221	1	IND	USA
42	*Amaranthus viridis*	238	1	PRI	USA
43	*Amaranthus hypochondriacus*	248	2	IND	USA
44	*Amaranthus hypochondriacus*	249	2	MEX	USA
45	*Amaranthus* sp.	388	2	THA	ITA
46	*Amaranthus viridis*	447	1	KOR	KOR
47	*Amaranthus retroflexus*	448	2	KOR	KOR
48	*Amaranthus caudatus*	450	1	ARG	MNG
49	*Amaranthus deflexus*	457	1	PRT	MNG
50	*Amaranthus crispus*	465	2	MEX	MNG
51	*Amaranthus viridis*	469	1	PER	MNG
52	*Amaranthus crispus*	479	2	MEX	MNG
53	*Amaranthus caudatus*	481	1	PER	MNG
54	*Amaranthus hybridus*	482	2	PER	MNG
55	*Amaranthus crispus*	484	2	MEX	MNG
56	*Amaranthus crispus*	486	2	MEX	MNG
57	*Amaranthus crispus*	499	2	PER	MNG
58	*Amaranthus hypochondriacus*	500	2	MEX	MNG
59	*Amaranthus caudatus*	505	2	MEX	MNG
60	*Amaranthus mantegazzianus*	535	1	ARG	RUS

Table 1. List of the 75 accessions and their model-based clusters. (continued)

No.[1)]	Species	Acc. No.[2)]	Inferred Cluster[3)]	Country of Origin	Provided Country
61	*Amaranthus mantegazzianus*	536	1	ARG	RUS
62	*Amaranthus* sp.	552	1	PER	RUS
63	*Amaranthus tricolor*	572	1	PRT	RUS
64	*Amaranthus mangostanus*	581	2	MEX	RUS
65	*Amaranthus mangostanus*	582	2	MEX	RUS
66	*Amaranthus caudatus*	588	2	BOL	RUS
67	*Amaranthus mangostanus*	593	2	MEX	RUS
68	*Amaranthus mangostanus*	594	2	MEX	RUS
69	*Amaranthus mangostanus*	605	2	MEX	RUS
70	*Amaranthus mangostanus*	615	1	PER	RUS
71	*Amaranthus mangostanus*	619	2	MEX	RUS
72	*Amaranthus mangostanus*	620	2	MEX	RUS
73	*Amaranthus tricolor*	625	2	ARG	UZB
74	*Amaranthus* sp.	639	1	BOL	RUS
75	*Amaranthus* sp.	640	1	BOL	RUS

[1)]Code number of the accessions for Structure analysis.
[2)]Final code number of the accessions in the National Genebank of the Rural Development Administration, Republic of Korea (RDA-Genebank).
[3)]Clusters based on structure result.

accessions from South Asia as controls) were obtained from the RDA-Genebank (Table 1).

SSR genotyping

DNA was extracted from fresh leaves of each accession using a Qiagen DNA extraction kit (Qiagen, Seoul, Republic of Korea). The relative purity and concentration of extracted DNA were estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). The final concentration of each DNA sample was adjusted to 20ng/ml. For SSR assays, a total of 14 polymorphic SSR markers were selected from those reported by Lee *et al*. (2008). To measure the size of PCR products, the M13-tail PCR method was used (Schuelke, 2000), as described previously (Lee *et al*., 2008). SSR alleles were resolved on an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, CA) using GeneScan 3.7 software (Applied Biosystems) and sized precisely using GeneScan 500 ROX (6-carbon-X-rhodamine) molecular size standards (35-500 bp) with the Genotyper 3.7 software (Applied Biosystems).

Data analysis

Using the genetic analysis package PowerMarker (ver. 3.23; Liu and Muse, 2005), variability at each locus was measured in terms of the number of alleles (NA), observed heterozygosity (H_O), major allele frequency (M_{AF}), gene diversity (GD), and polymorphic information content (PIC). The PIC value can be used to evaluate diversity using the formula:

$$PIC_l = 1 - \sum_{i=1}^{n} p_{il}^2$$

where p_{il} represents the allele frequency of the i^{th} allele at locus *l*. The shared allele frequencies were also calculated to measure genetic distances between each pair of accessions using PowerMarker (ver. 3.23). The neighbor-joining method was used to construct a phylogram from a distance matrix using the MEGA4 software (Tamura *et al*., 2007) embedded in PowerMarker. Same software was used to test the Hardy-Weinberg equilibrium (HWE) and pairwise linkage disequilibrium (LD).

The possible population structure was analyzed using the model-based software program Structure 2.3.3 (Pritchard *et al*., 2000; Falush *et al*., 2003) in the 75 amaranth accessions and a model allowing admixture and correlated allele frequencies. At least four runs of Structure were performed with the number of clusters (*K*) set from 1 to 10, and the average likelihood value, L(*K*), across all runs was calculated for each *K*. In this model, several clusters (*K*) are assumed to be present, each of which is characterized by a set of allele frequencies for each locus. Individuals in the sample were assigned to clusters or jointly to two or more populations if their genotypes indicated that they were admixed. The model choice criterion to detect the most likely value of *K* was ΔK, which is an *ad hoc* quantity related to the second-order change in the log probability of data (LnP[D]) with respect to the number of clusters inferred by Structure (Evanno *et al*., 2005). An individual was assigned to a group if > 75% of its genome fraction value was derived from that group.

The molecular variance between two clusters resulting from model-based population and F_{ST}, the correlation of alleles within populations, were calculated by the analysis of molecular variance (AMOVA) approach in the Arlequin 3.11 program (Schneider and Excoffier, 1999; Excoffier and Schneider, 2005).

RESULTS

SSR polymorphism in the entire sample

Using the PowerMarker software (ver. 3.23; Liu and Muse, 2005), SSR polymorphisms were measured in terms of the numbers of alleles, gene diversity, and PIC. The 14 SSR markers revealed 99 alleles among the 75 amaranth accessions of 65 were from South America and 10 from South Asia with allele size ranging from 114 bp (132F) to 256 bp (51F). The allelic richness per locus varied widely among the markers, ranging from 2 (78N) to 14 (132F), with an average of seven alleles per locus (Table 2). The

Table 2. Size range, number of alleles, number of rare alleles, major allele frequency, genetic diversity, and polymorphism information content index for 14 SSR loci in 75 accessions.

Marker	Size range	NA[a]	Rare alleles	M_{AF}[b]	GD[c]	PIC[d]
13F	171-178	4	2	0.62	0.50	0.42
32N	164-176	5	1	0.59	0.51	0.48
51F	247-256	4	1	0.44	0.67	0.60
123F	203-245	5	3	0.43	0.45	0.39
104H	190-247	9	5	0.31	0.53	0.51
57N	130-303	3	1	0.56	0.20	0.19
129H	179-343	8	4	0.20	0.70	0.67
71N	175-184	4	1	0.62	0.56	0.46
132F	114-177	16	10	0.29	0.85	0.83
137H	212-245	11	6	0.26	0.81	0.79
78N	115-167	2	1	0.93	0.03	0.03
99N	140-185	15	8	0.16	0.89	0.88
105N	124-172	6	2	0.36	0.73	0.69
136N	204-225	7	4	0.38	0.63	0.56
Total		99	49	6.15	8.05	0.53
Mean		7	3.5	0.44	0.58	0.42

[a] Number of alleles.
[b] Major allele frequency.
[c] Genetic diversity.
[d] Polymorphic information content.

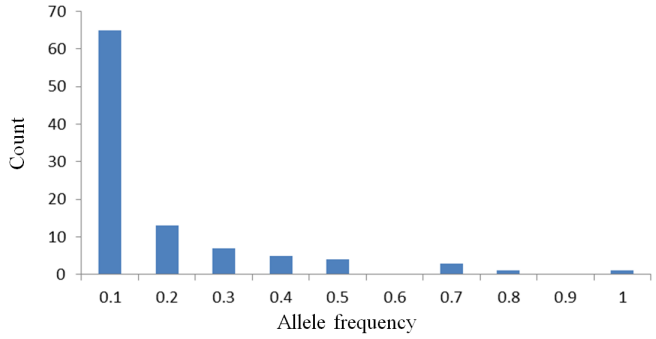

Fig. 1. Histogram of allele frequencies for all 99 alleles in the 75 amaranth accessions.

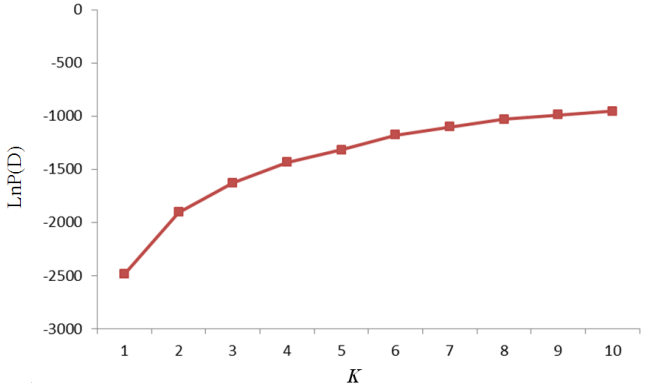

Fig. 2. (Log) Likelihood of the data (n = 75), $L(K)$, as a function of K (number of clusters used to stratify the sample).

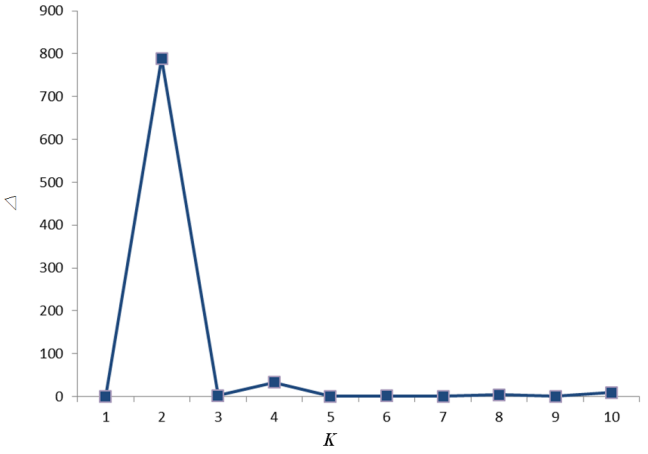

Fig. 3. *Values of ΔK, with its modal value detecting the true K of two clusters* (K = 2).

database of allelic frequencies showed that rare alleles (frequency < 0.05) comprised 49.50% of all alleles, whereas intermediate (frequency 0.05–0.50) and abundant alleles (frequency > 0.50) comprised 45.45% and 5.05%, respectively, of all alleles detected. These results indicated the presence of a relatively large proportion of rare alleles, and most alleles were present at low frequency among the amaranth accessions studied (Table 2, Fig. 1). The genetic diversity varied from 0.03 (78N) to 0.89 (99 N), with an average of 0.58. A strong correlation was found between gene diversity and allelic richness (r = 0.77, P < 0.01, data not shown), as reported previously (Herrera *et al*., 2008). PIC values ranged from 0.03 to 0.88, with an average of 0.42 (Table 2).

Genetic diversity and population structure analysis

To determine the population structure of 75 accessions, the model-based program Structure was used with all accessions and 14 SSR loci. The distribution of L(K) did not indicate a clear mode for the true K (Fig. 2). Therefore, a further *ad hoc* quantity (ΔK) was used to overcome the difficulty of interpreting real K values (Evanno *et al*., 2005). The true value of K can be determined by illustration of the peak based on ΔK. A highest peak of ΔK for 75 accessions was found for K = 2 (Fig. 3). At K = 2, the highest number of accessions was assigned to one specific cluster with a probability higher than 96%, so it was used for the final analysis. The relatively small value of the alpha parameter (α = 0.0429) at K = 2 indicated that most of the accessions originated from one primary ancestor, with a few admixed individuals (Ostrowski *et al*., 2006).

Of the 75 amaranth accessions included in the analysis, 72 (96%) shared > 75% membership with one of two clusters and were classified as members of that cluster, whereas three accessions (4%) were categorized as admixture forms with varying levels of membership shared between the two clusters. Cluster 1 consisted of 41 accessions (11 species) and the remaining 31 accessions (13 species) were classified as cluster 2 (Table 1, Fig. 3). The mean gene diversities for each SSR locus in Cluster 1 and Cluster 2 of amaranth were 0.47 and 0.40, respectively, and the mean PIC values for each SSR locus were 0.43 and 0.37, respectively. The comparative study of genetic diversity showed that accessions from Cluster 1 (GD = 0.47, PIC = 0.43) possessed greater genetic diversity than Cluster 2 (GD

Table 3. Diversity information and F_{ST} values of the two clusters.

Inferred cluster	Diversity					F_{ST} [e]		
	Sample size	NA[a]	M_{AF}[b]	GD[c]	PIC[d]	1	2	Overall
1	41	4.79	0.66	0.47	0.43	0.000		
2	31	4.07	0.70	0.40	0.37	0.314	0.000	0.3630
Average		4.43	0.68	0.44	0.40			

[a] Number of alleles.
[b] Major allele frequency.
[c] Gene diversity.
[d] Polymorphic information content.
[e] For AMOVA-based estimates, P<0.005 for 100 permutations for all population comparisons.

Table 4. Analysis of molecular variance (AMOVA) of a number of populations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage variation	*p*
Model-based population					
Among clusters	1	122.8	1.8	36.3	< 0.05
Within	72	416.6	3.2	63.7	
Geographic Origin					
Among clusters	1	9.4	0.1	1.00	<0.05
Within	72	531.5	4.1	99.00	

= 0.40, PIC = 0.37) (Table 3). However, the average allele number in Cluster 2 (0.70) was higher than that in Cluster 1 (0.66), with an average of 0.68 for the two clusters. The average genetic distances were also used to evaluate the genetic diversities of the species. The average genetic distance for Cluster 1 (0.6477) was greater than Cluster 2 (0.5233), indicating that the Cluster 1 accessions had a higher level of genetic variation than that of Cluster 2. In this study, both the average number of alleles and mean alleles per locus of Cluster 1 was higher than that of Cluster 2. The distribution of molecular genetic variation among and within clusters was estimated by AMOVA, which revealed that 36.3% of the total variation was among clusters, and 63.7% of the variation was within clusters. When 75 accessions were compared by AMOVA across geographic regions (two groups: South America and South Asia), 1% of the total variation was observed between clusters, and 99% of the variation was within clusters (Table 4).

The overall F_{ST} value was 0.3630, indicating moderate differentiation between the two clusters. Comparison of the levels of polymorphism in the two defined clusters showed how genetic diversity was organized (Table 3). When the genetic backgrounds of two clusters were compared, Cluster 1 was found to be comprised of 41 accessions with higher average allele numbers (4.79) and polymorphism levels (0.43).

DISCUSSION

In the present study, 65 accessions from South American and 10 accessions from South Asia were estimated the genetic diversity by using 14 SSR markers. Structure analysis revealed two clusters and three admixtures (Table 1, Fig. 4). Cluster 1 included 41 accessions representing 11 species: 10 from South America and only 1 species from South Asia (Table 1). Among 11 species in this cluster, seven species (*Amaranthus blitum*, *Amaranthus deflexus*, *Amaranthus graecizans* subsp. *sylvestris*, *Amaranthus mantegazzianus*, *Amaranthus standleyanus*, *Amaranthus viridis*, *Amaranthus quitensis*) were found in only Cluster 1, and four species (*Amaranthus caudatus*, *Amaranthus*

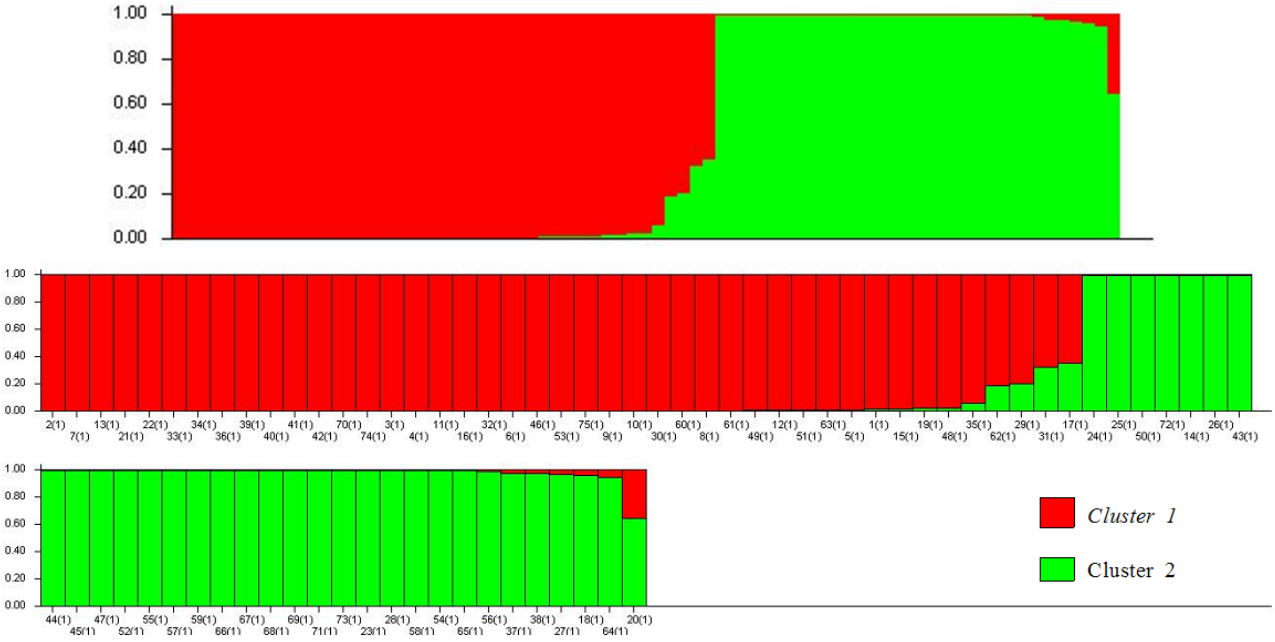

Fig. 4. Model-based ancestry for each of the 75 accessions based on the 14 SSR markers used to build the Q matrix. The numbers represent the serial numbers of the accessions. The numbers in parentheses are predefined cluster numbers.

hybridus, *Amaranthus* sp., and *Amaranthus tricolor*) were also found in Cluster 2. Cluster 2 had 31 accessions representing 14 species. The highest level of genetic diversity (0.89) was observed with loci 99N, the lowest level of genetic diversity value (0.03) was observed with loci 78N, and the mean diversity was 0.58 (Nei 1973). It was noted that a marker detecting a lower number of alleles also showed lower genetic diversity, compared to markers detecting higher numbers of alleles, which revealed higher levels of genetic diversity. The maximum number of repeats within the SSRs was also positively correlated with the genetic diversity.

The values of pairwise comparisons of Nei's genetic distance (D) between the genotypes were computed from combined data for 14 primers (Nei 1973). Comparative analysis of genetic variation between the two clusters indicated that the average genetic distance of Cluster 1 (0.6477) was higher than that of Cluster 2 (0.5233). This indicated that the accessions included in Cluster 1 had a higher level of genetic variation than those in Cluster 2. On the other hand, a smaller genetic distance (0.5233) was found in Cluster 2, indicating that these species were genetically much closer. The genetic distances between

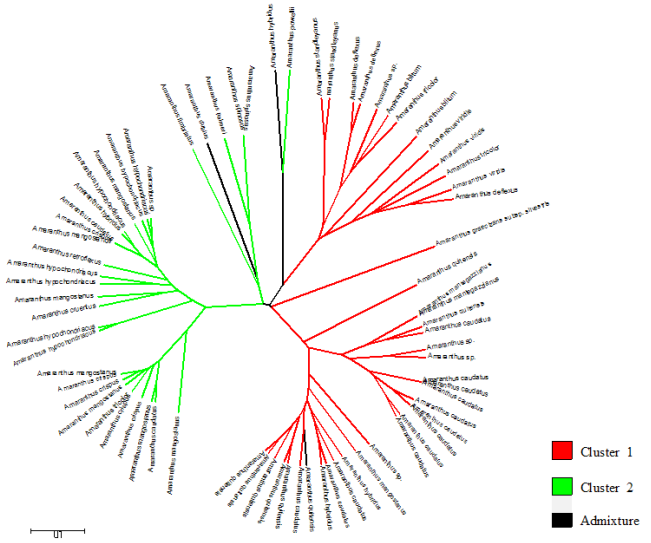

Fig. 5. Unrooted neighbor-joining tree (UPGMA) based on Nei's genetic distance matrix (shared allele frequency) among 75 amaranth accessions. Colors correspond to those of the model-based populations.

accessions of Clusters 2 revealed a close relationship.

The genetic distance-based results observed in the unrooted neighbor-joining tree revealed a similar trend to the genetic similarity analysis using model-based Structure (Fig. 5).

The occurrence of some admixed/hybrid genotypes indicated frequent hybridization/ introgression events. Although the extent and significance of natural hybridization/ introgression are unclear, new gene combinations of domestic cultivars and their wild or weedy relatives are important for the evolution of domesticated plant species (Jarvis and Hodgkin, 1999). Amaranth shows a wide variety of morphological diversity among and even within certain species. Although the family (Amaranthaceae) is distinctive, the genus has few characteristics that distinguish the species. This complicates taxonomy, and *Amaranthus* is generally considered a "difficult" genus.

Cluster 1 showed greater genetic diversity than Cluster 2 (Table 3). The results of AMOVA of geographic regions (South America and South Asia) showed only 1% variation among the two groups, indicating that it was not possible to identify the origin relatedness of the two populations as no correlations between origin and subpopulations were found. This may be due to the cosmopolitan nature of the genus, *Amaranthus*, and the results of human activities, such as breeding and resource exchange. This result agreed with the finding of Khaing et al. (2013). Of the total of 21 species used in this study, four (*Amaranthus caudatus*, *Amaranthus tricolor*, *Amaranthus hybridus*, and *Amaranthus* sp.) were found in both clusters. These species are well-known grains (*Amaranthus caudatus* and *Amaranthus hybridus*) and leafy vegetable and ornamental species (*Amaranthus tricolor*). These results indicated complex genetic relationships between the species.

In conclusion, 99 alleles were detected with an average of 7.07 alleles per SSR locus among the whole amaranth collection (75 accessions). Model-based Structure analysis revealed the presence of two clusters, which was essentially consistent with clustering based on the GD. Assessment of genetic diversity is an essential component of germplasm characterization and conservation. The results derived from analyses of genetic diversity could be used to design effective breeding programs with the aim of broadening the genetic bases of commercially grown varieties. However, these results must be compared with a wider range of *Amaranthus* sp., which will facilitate the effective use and conservation management of *Amaranthus* germplasm for crop improvement.

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