韓藥作誌(Korean J. Medicinal Crop Sci.) 21(3) : 220 – 228 (2013)
ISSN(Print) 1225-9306
ISSN(Online) 2288-0186
http://www.medicinalcrop.org
http://dx.doi.org/10.7783/KJMCS.2013.21.3.220

SSR 마커를 이용한 남아시아와 동남아시아 아마란스 자원의 유전적 다양성 비교

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Comparison of Genetic Diversity among Amaranth Accessions from South and Southeast Asia using SSR Markers

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ABSTRACT : This study was conducted to assess the genetic diversity and population structure of 70 amaranth accessions collected from South and Southeast Asia using 14 simple sequence repeat (SSR) markers. In total, 67 alleles were detected, with an average of 4.79 per locus. Rare alleles comprised a large portion (46.3%) of the detected alleles, and 29 unique alleles associated with rice accessions were also discovered. The mean major allele frequency (MAF), genetic diversity (GD) and polymorphic information content (PIC) of the 14 SSR loci were 0.77, 0.36, and 0.34, respectively. A model-based structural analysis revealed the presence of three subpopulations. The genetic relationships revealed by the neighbor-joining tree method were fairly consistent with the structure-based membership assignments for most of the accessions. All 70 accessions showed a clear relationship to each cluster without any admixtures. We observed a relatively low extent of genetic exchange within or among amaranth species from South and Southeast Asia. The genetic diversity results could be used to identify amaranth germplasms and so facilitate their use for crop improvement.

Key Words **:** Amaranth, Genetic Diversity, Population Structure, SSRs

INTRODUCTION

Amaranthus, planted mainly for its grain and leaves, is cultivated in many tropical and temperate regions worldwide (Tucker, 1986). The genus *Amaranthus* includes more than 60 species, but not all are found in daily menus. Amaranth species such as *Amaranthus blitus*, *Amaranthus cruentus*, *Amaranthus hypochondriacus* are often planted for leaves, whereas *Amaranthus caudatus*, *Amaranthus hypochodriacus, Amaranthus cruentus, Amaranthus hybridus* are planted for their grain (Caselato-Sousa and Amaya-Farfn, 2012). Amaranth was consumed as a staple food during ancient times. Ancient amaranth grains still in use today include *Amaranthus caudatus, Amaranthus cruentus*, and *Amaranthus hypochondriacus*; their cultivation is expanding in Central and South America, Africa, and some parts of Asia. In recent years, amaranth has gained attention as a food and fodder crop due to its high seed protein content, balanced amino acid composition, and high lysine content (Caselato-Sousa and Amaya-Farfn, 2012; Zheleznov *et al*., 1997).

Amaranth leaves are a rich yet inexpensive source of dietary fiber, protein, vitamins and a wide range of minerals. Vegetable amaranth is an indispensable source of nutrition, especially protein for vegetarian people in developing countries. Vegetable and grain *Amaranthus* species are tolerant to infestation by herbivorous insects under field conditions, and *Amaranthus* can grow successfully under varied soil and agro-climatic conditions (Angel and Paulina,

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Received 2013 April 15 / 1st Revised 2013 May 14 / 2nd Revised 2013 June 7 / Accepted 2013 June 7

2011; Brenner *et al*., 2010; Prakash and Pal, 1991). Currently, grain amaranths are cultivated in many parts of the world, including Central and South America, Africa, India and China. Amaranth grain can be cooked, popped, toasted, extruded or milled for consumption. In India, food containing amaranth grain can be a succedaneum to wheat and it is easy to incorporate into traditional cuisine (Dixit *et al*., 2011; Pandey and Singh, 2010).

The medicinal importance of this crop has been known for a long time. Amaranth is a rich dietary source of β-sitosterol and other phytosterols. It has been clearly shown that consumption of phytosterols, plant sterols, significantly reduce plasma cholesterol levels without causing noticeable side effects (Marcone *et al*., 2003). Amaranth oil is applicable as an effective natural antioxidant supplement, which could be of significant benefit for patients with cardiovascular disease, due to the high content of unsaturated fatty acids and unique presence of squalene (Martirosyan *et al*., 2007). Tea with the leaves of amaranth for prophylactic and therapeutic purposes was developed in Russia (Kononkov *et al*., 2007). Amaranth was applied as means of cleaning the stomach, a diuretic, to cure intestinal colic, cough, headaches and tumors (Ofitserov, 2001). These curative effects are often attributed to different antioxidant components. Antioxidant activities of the amaranth seed extract positively correlated with the presence of total polyphenols (Gorinstein *et al*., 2007). Flavonoids as one of the natural antioxidants are considered to be effective substances for the prevention of diseases of the higher age. Flavonoids in amaranth, such as rutin and quercetin, are important antioxidants that significantly inhibit the oxidation of high density lipoprotein (HDL) cholesterol. This crop, therefore, is one of the new world super grains and is gaining favor among health-conscious consumers in many countries.

Information on the genetic diversity and relationships within and among crop species is essential for the efficient utilization of plant genetic resource collections (Brown, 1989). Various species of the genus *Amaranthus* L. can be used as donors of economically valuable genes in the selection of amaranth. These species of amaranth are also of great interest for the development of grain cultivars (Limanskii, 2012). The involvement of various genotypes in genetic selection programs of *Amaranthus* requires a detailed investigation of this crop. Genebanks, which served as the major means of genetic resource conservation, encounter problems such as superfluous conservation of crop varieties and the increasing cost of maintenance. Nowadays, core sets represent a more efficient approach to preserving genetic diversity and minimizing sample collection (Brown, 1989; Frankel, 1984; Kim *et al*., 2007). A core set of *Amaranthus* has been developed from a total of 634 accessions and its relatives conserved using a heuristic approach (Khaing *et al*., 2013).

DNA molecular markers based on PCR have proved to be a useful and informative tool for estimating the genetic diversity and genetic relationships in crop germplasm (Gwag *et al*., 2010; Li and Park, 2012; Zhao *et al*., 2010; Zhao *et al*., 2011). Several molecular approaches have been employed to assess genetic diversity in *Amaranthus*. Chan and Sun (1997) analyzed the genetic diversity and relationships of 23 cultivated and wild *Amaranthus* species using random amplified polymorphic DNA (RAPD) markers. Amplified fragment length polymorphism (AFLP) DNA markers have been used to determine the genetic relationship among weedy *Amaranthus* species (Wassom and Tranel, 2005). SSR markers have been shown to be a particularly powerful tool for this kind of research because of their abundance in eukaryotic genomes, co-dominance, and high frequency of polymorphisms (Gwag *et al*., 2010; Moe *et al*., 2010; Zhao *et al*., 2012). SSR has also been applied to evaluation of the genetic diversity and population structure in *Amaranthus* (Khaing *et al*., 2013). The purpose of the current study was to determine the population genetic structure of *Amaranthus* accessions from South and Southeast Asia.

MATERIALS AND METHODS

1. Plant materials

In this study, 70 amaranth accessions from South and Southeast Asia were selected. Of them, 25 accessions were from India, 32 from Nepal, and 13 from Thailand (Table 1). All plant materials collected were obtained from the National Genebank of the Rural Development Administration, Republic of Korea (RDA-Genebank). Total DNA was extracted from all accessions using a Qiagen DNA extraction kit (Qiagen, Seoul, Korea).

Table 1. List of the 70 amaranth accessions used in this study and their model-based groupings.

Serial No	species	Countries of origin	Region	Subpopulation ownership*
1	*Amaranthus blitum*	India	South Asia	S1
2	*Amaranthus blitum*	India	South Asia	S1
3	*Amaranthus blitum*	India	South Asia	S1
4	*Amaranthus blitum*	India	South Asia	S1
5	*Amaranthus blitum*	India	South Asia	S1
6	*Amaranthus blitum*	India	South Asia	S1
7	*Amaranthus blitum*	India	South Asia	S1
8	*Amaranthus caudatus*	India	South Asia	S2
9	*Amaranthus caudatus*	India	South Asia	S2
10	*Amaranthus caudatus*	India	South Asia	S2
11	*Amaranthus caudatus*	India	South Asia	S2
12	*Amaranthus caudatus*	India	South Asia	S2
13	*Amaranthus caudatus*	India	South Asia	S3
14	*Amaranthus hypochondriacus*	India	South Asia	S3
15	*Amaranthus hypochondriacus*	India	South Asia	S3
16	*Amaranthus hypochondriacus*	India	South Asia	S3
17	*Amaranthus hypochondriacus*	India	South Asia	S3
18	*Amaranthus hypochondriacus*	India	South Asia	S3
19	*Amaranthus hypochondriacus*	India	South Asia	S3
20	*Amaranthus hypochondriacus*	India	South Asia	S3
21	*Amaranthus hypochondriacus*	India	South Asia	S3
22	*Amaranthus caudatus*	India	South Asia	S3
23	*Amaranthus caudatus*	India	South Asia	S3
24	*Amaranthus caudatus*	India	South Asia	S3
25	*Amaranthus caudatus*	India	South Asia	S3
26	*Amaranthus hypochondriacus*	Nepal	South Asia	S3
27	*Amaranthus hypochondriacus*	Nepal	South Asia	S3
28	*Amaranthus hypochondriacus*	Nepal	South Asia	S3
29	*Amaranthus hypochondriacus*	Nepal	South Asia	S3
30	*Amaranthus hypochondriacus*	Nepal	South Asia	S3
31	*Amaranthus hypochondriacus*	Nepal	South Asia	S3
32	*Amaranthus hypochondriacus*	Nepal	South Asia	S3
33	*Amaranthus* sp.	Nepal	South Asia	S3
34	*Amaranthus* sp.	Nepal	South Asia	S3
35	*Amaranthus* sp.	Nepal	South Asia	S3
36	*Amaranthus* sp.	Nepal	South Asia	S3
37	*Amaranthus* sp.	Nepal	South Asia	S3
38	*Amaranthus* sp.	Nepal	South Asia	S3
39	*Amaranthus* sp.	Nepal	South Asia	S3
40	*Amaranthus* sp.	Nepal	South Asia	S3
41	*Amaranthus* sp.	Nepal	South Asia	S2
42	*Amaranthus* sp.	Nepal	South Asia	S3
43	*Amaranthus* sp.	Nepal	South Asia	S3
44	*Amaranthus* sp.	Nepal	South Asia	S3
45	*Amaranthus* sp.	Nepal	South Asia	S3
46	*Amaranthus* sp.	Nepal	South Asia	S3
47	*Amaranthus* sp.	Nepal	South Asia	S3
48	*Amaranthus* sp.	Nepal	South Asia	S3
49	*Amaranthus* sp.	Nepal	South Asia	S2
50	*Amaranthus* sp.	Nepal	South Asia	S3

Table 1. List of the 70 amaranth accessions used in this study and their model-based groupings. (Continued)

Serial No	species	Countries of origin	Region	Subpopulation ownership*
51	*Amaranthus* sp.	Nepal	South Asia	S3
52	*Amaranthus* sp.	Nepal	South Asia	S3
53	*Amaranthus* sp.	Nepal	South Asia	S2
54	*Amaranthus* sp.	Nepal	South Asia	S2
55	*Amaranthus* sp.	Nepal	South Asia	S3
51	*Amaranthus* sp.	Nepal	South Asia	S3
52	*Amaranthus* sp.	Nepal	South Asia	S3
56	*Amaranthus* sp.	Nepal	South Asia	S3
57	*Amaranthus* sp.	Nepal	South Asia	S3
58	*Amaranthus* sp.	Thailand	Southeast Asia	S3
59	*Amaranthus* sp.	Thailand	Southeast Asia	S2
60	*Amaranthus* sp.	Thailand	Southeast Asia	S3
61	*Amaranthus* sp.	Thailand	Southeast Asia	S3
62	*Amaranthus* sp.	Thailand	Southeast Asia	S3
63	*Amaranthus* sp.	Thailand	Southeast Asia	S3
64	*Amaranthus* sp.	Thailand	Southeast Asia	S2
65	*Amaranthus* sp.	Thailand	Southeast Asia	S3
66	*Amaranthus* sp.	Thailand	Southeast Asia	S3
67	*Amaranthus* sp.	Thailand	Southeast Asia	S3
68	*Amaranthus* sp.	Thailand	Southeast Asia	S3
69	*Amaranthus* sp.	Thailand	Southeast Asia	S3
70	*Amaranthus* sp.	Thailand	Southeast Asia	S3

*As defined by the STRUCTURE software.

2. SSR genotyping

Fourteen polymorphic SSR markers developed by Lee *et al.* (2008) were used in this study. A three-primer system (Schuelke, 2000) was used. This included a universal M13 oligonucleotide (TGTAAAACGACGGCCAGT) labeled with one of three fluorescent dyes (6-FAM, NED, or HEX), which allowed the products to be triplexed during electrophoresis; a special forward primer composed of a concatenation of the M13 oligonucleotide, and the normal reverse primer for SSR PCR amplification. The SSR alleles were resolved on an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the GeneScan 3.7 software, 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).

3. Data analysis

The major allele frequency (*MAF*), number of alleles, genetic diversity (*GD*), and polymorphic information content (*PIC*) value were determined using the genetic analysis package PowerMarker ver. 3.25 (Liu and Muse, 2005). The *PIC* value can be used to evaluate diversity effectively 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 genetic distance among accessions was calculated as Nei's distance (Nei *et al*., 1983) using the neighbor-joining method, and an unrooted phylogram was constructed using the MEGA4 software as implemented in PowerMarker (Tamura *et al*., 2007). Genetic distances between groups of varieties, as per Nei and Li (1979), were calculated using the equation;

$$GD_{XY} = 1 - \left(\frac{2N_{XY}}{[N_X + N_Y]}\right)$$

where N_X and N_Y represent the number of alleles in groups X and Y, respectively, and N_{XY} is the number of alleles shared between the two groups. The model-based

software STRUCTURE 2.2 (Schuelke, 2000) was used to identify the population structure of the accessions using a Bayesian clustering approach. Four independent replicates were performed per run, with *K* ranging from 2 to 8 with a burn-in of 10,000 and run length of 50,000. The most probable number (*K*) was calculated based on the method of Flint-Garcia *et al*. (2003) using a model allowing for admixtures and correlated allele frequencies. An inferred ancestry of ≥ 75% was used to assign rice accessions of the same subgroup, while < 75% was assigned to an admixture group.

RESULTS

1. Overall SSR diversity

A total of 14 SSR markers were used to assess the genetic diversity and population structure among 70 amaranth accessions (Table 2). All markers were polymorphic across the 70 amaranth accessions, and 67 alleles were identified. Of the 67 alleles, 23 (34.3%) were common (frequency 0.05 - 0.5); 31 (46.3%) were rare (frequency < 0.05); and 13 (19.4%) were abundant (frequency > 0.5). These results reveal a large proportion of rare alleles among the accessions studied (Fig. 1). The number of alleles detected per locus ranged from 2 to 11, with an average of 4.79 per locus, whereas the number of rare alleles identified among these loci varied from 0 to 19, with a mean of 2.21 per locus. Sixteen rare alleles were found exclusively in single accessions. The major allele frequency (*MAF*) ranged from 0.53 to 0.98 with a mean of 0.77. The genetic diversity (*GD*) and polymorphism information content (*PIC*) varied from 0.03 to 0.66 and 0.03 to 0.62, with an average of 0.66 and 0.62, respectively (Table 2).

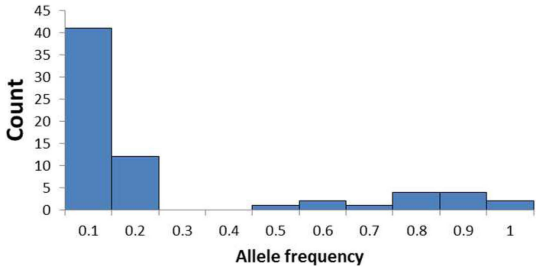

Fig. 1. Histograms of the frequencies of the 67 alleles in 70 accessions of amaranth and its relatives.

2. Geographical analysis of diversity

As shown in Table 3, among the accessions used in this study, the average number of alleles followed the order Nepal > India > Thailand. A countrywide comparative study of genetic diversity showed that accessions from India possessed the highest genetic diversity (*GD* = 0.56, *PIC* = 0.52), followed by Nepal (*GD* = 0.2, *PIC* = 0.18) and Thailand (*GD* = 0.18, *PIC* = 0.14). It is worth mentioning

Table 2. Overall diversity statistics for 14 SSR loci in 70 amaranth accessions.

Locus	Size range (bp)	*NA*[a]	*NA*[b]	*MAF*[c]	*GD*[d]	*PIC*[e]
13F	171-175	3	1	0.83	0.30	0.28
32N	167-173	3	0	0.74	0.42	0.38
51F	247-256	4	1	0.73	0.43	0.40
57N	130-303	2	1	0.98	0.03	0.03
71N	175-181	2	0	0.90	0.18	0.16
78N	111-167	3	2	0.96	0.08	0.08
99N	155-182	6	2	0.70	0.49	0.46
104H	190-235	5	3	0.86	0.26	0.25
105N	151-172	5	1	0.70	0.48	0.44
123F	203-239	4	2	0.87	0.24	0.23
129H	179-262	6	2	0.60	0.60	0.56
132F	114-162	11	9	0.59	0.61	0.58
136N	156-222	6	4	0.84	0.29	0.27
137H	218-239	7	3	0.53	0.66	0.62
Total		67	31			
Mean		4.79	2.21	0.77	0.36	0.34

A; Number of alleles, b; Number of rare alleles, c; Major allele frequency, d; Gene diversity, e; Polymorphism information content.

Table 3. Number of amaranth accessions, number of alleles, major allele frequency, genetic diversity, and polymorphic information content according to region/country.

Region	Country	NA[a]	*NA*[b]	*MAF*[c]	*GD*[d]	*PIC*[e]
South Asia	India	25	4.21	0.57	0.56	0.52
	Nepal	32	2.50	0.88	0.20	0.18
Southeast Asia	Thailand	13	1.64	0.90	0.16	0.14

A; Number of amaranth accessions, b; Number of alleles, c; Major allele frequency, d; Gene diversity, e; Polymorphism information content.

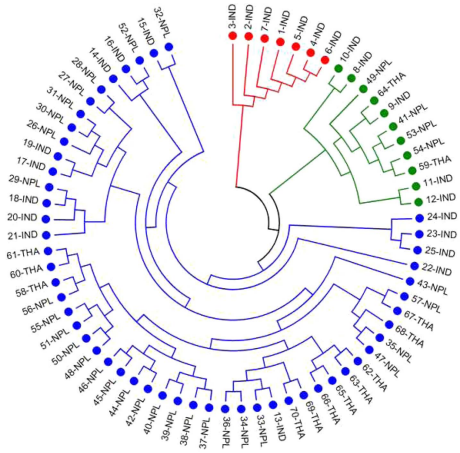

Fig. 2. An unrooted neighbor-joining tree showing the genetic relationships among the 70 amaranth accessions based on 14 microsatellite markers. The color corresponds to that of model-based populations.

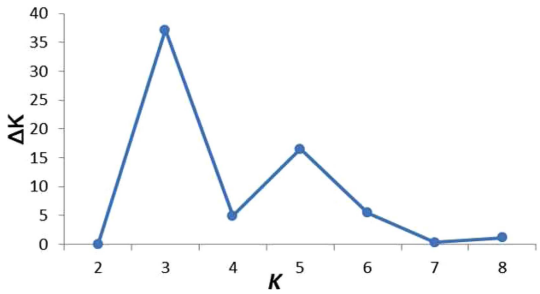

Fig. 3. Magnitude of *ΔK* as a function of *K*. In this case, the maximum value of *ΔK* of the 70 amaranth accessions was identified at $K = 3$.

that accessions from India possessed relatively high *PIC* values and a large number of alleles with a comparative small number of accessions.

3. Distance-based phylogeny

A genetic distance-based analysis was performed by calculating the shared allele frequencies among the 70 accessions. An unrooted phylogram was computed using MEGA 4 (Tamura *et al*., 2007) embedded in the PowerMarker program (Liu and Muse, 2005). The UPGMA tree clustered all of the accessions into three groups. As shown in Fig. 2, 70 amaranth accessions were distributed among the three groups. The first group included seven accessions from India. The second group consisted of 11 accessions, five of them from India, four from Nepal, and two from Thailand. The third group is the main group, which included about 75% of the accessions used in this study; the numbers of accessions from India, Nepal and Thailand distributed in this group were 13, 28 and 11, respectively.

Table 4. Distribution of accessions from different countries to each population identified by the inferred value from the STRUCTURE software.

Region	Country	S1	S2	S3	Total
South Asia	India	7	5	13	25
	Nepal	0	4	28	32
Southeast Asia	Thailand	0	2	11	13
		7	11	52	70
Total		(10%)	(16%)	(74%)	

4. Population structure

Population structure analysis was carried out using the STRUCTURE 2.2 software (Pritchard *et al*., 2000), which implements a Bayesian approach to identify subpopulations with distinct allele frequencies and places individuals into K clusters. The distribution of L (K) revealed a continuously increasing curve without a clear maximum for the true K, although K did show a clear peak at the true value of $K = 3$ (Fig. 3), indicating that the accessions could be grouped into three main subpopulations (Evanno *et al*., 2005).

All 70 accessions shared > 75% membership with one of the genetic populations and were classified as members of that population without admixture (Table 4, Fig. 4). As

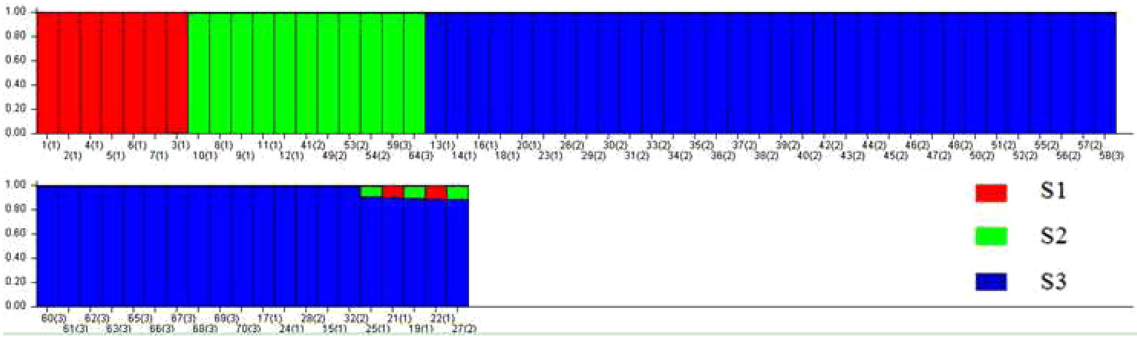

Fig. 4. Model-based clustering for each of the 70 amaranth accessions examined based on 14 SSR markers used to build the Q matrix. Each individual bar represents an accession. The color bars refer to three genetic groups (S1-S3, respectively).

shown in Figure 4, the distribution of 70 amaranth accessions classified by STRUCTURE was inconsistent with that of UPGMA tree. Accessions from India were distributed in all three subgroups, whereas accessions from Nepal and Thailand were found only in groups two and three, respectively.

DISCUSSION

Genetic diversity is critical in crop breeding. Strong genetic diversity provides diverse morphological traits and potentially valuable genetic information, and therefore lays a good foundation for breeding (Upadhyay *et al*., 2012). Microsatellites have become one of the most widely used molecular markers for genetic diversity studies, linkage map construction, and marker-assisted selection (MAS) (Chung *et al*., 2009; Zhao *et al*., 2011).

The objective of this study was to assess the genetic diversity in selected amaranth accessions using SSR markers. Abundant allelic variation was discovered, with an average of 4.79 per locus. A considerable number of rare alleles were identified, which comprised a large proportion of the total, indicating that rare alleles make a major contribution to the overall genetic diversity of the germplasm (Roussel *et al*., 2004; Yifru *et al*., 2006). More to the point, sixteen unique alleles were exclusive to single accessions, which may be associated with special traits and could be useful in further gene identification and MAS breeding.

Genetic advancement can be achieved by artificial selection based on valuable agronomic traits controlled by genetic inheritance. A number of studies have focused on genetic divergence and its association with agronomic traits. Wu *et al*. (2000) identified amaranth genotypes that carry valuable agronomic traits, such as grain yield and disease tolerance, and non-cultivated species were reported to be more tolerant to disease. Pandey and Singh (2010) found genotypes significantly associated with leaf protein content in grain amaranth. Due to extensive distribution and multiple sub-species, favorable agronomic traits could vary among regions because of human activities related to selection and resource exchange, which may result in genetic divergence among areas (Kumar *et al*., 2010).

The neighbor-joining tree of 70 amaranth accessions based on Nei's (1983) genetic distance revealed that the rice materials used could be divided into three distinct groups, with the major group consisting of more than 70% of the accessions. Some accessions of the same species scattered to different groups such as *Amaranthus caudatus* and *Amaranthus sp.,* despite coming from the same country. Accessions from India showed more genetic diversity than those from Nepal and Thailand; this may be due in part to the divergence origin of the region in which they were collected. Worldwide collections will be critical for the enrichment of the genetic diversity of amaranth germplasm. Although a correlation between origin and subpopulations was not found in this study, one of the species, *Amaranthus blitum* from India, clustered in a singular subpopulation.

Understanding the population structure is vital to confirm the correlation between phenotype and genotype, and is a precondition for appropriate selection of accessions. The results of our structure analysis and neighbor-joining tree were in good agreement. Accessions distributed in the same subpopulations by STRUCTURE 2.2 were clustered

together in the dendrogram (Fig. 4). High levels of inter-accessional genetic diversity were found within species, but genetic uniformity was observed within most accessions. All of the accessions were clustered into three groups without admixture, indicating relatively rare genetic communication among those amaranth species. Only *Amaranthus caudatus* and *Amaranthus sp.* were distributed in more than one subgroup, which suggests that these two species have comparatively high genetic divergence than other species in this study. Slight genetic exchange was identified between species *Amaranthus hypochondriacus* and *Amaranthus blitum*, and *Amaranthus hypochondriacus* and *Amaranthus caudatus* among few accessions from India and Nepal.

In conclusion, SSR markers are an effective tool for identifying the genetic variability in amaranth collections. We found a relatively low degree of genetic exchange within or among amaranth species from South and Southeast Asia.

ACKNOWLEDGEMENTS

This work was supported by a grant from the BioGreen 21 Program(No. PJ009099), Rural Development Administration, Republic of Korea.

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