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Analysis of Genetic Diversity and Population Structure of Buckwheat (*Fagopyrum esculentum* Moench) Landraces of Korea Using SSR Markers

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Abstract - Buckwheat (*Fagopyrum esculentum* Moench), one of the minor crops grown in Korea belonging to the Polygonaceae family, is an annual crop widely cultivated in Asia, Europe, and America and has a character of outcrossing and self-incompatibility. The objective of this study was to analyze the genetic variability, phylogenetic relationships and population structure of buckwheat landraces of Korea using SSR markers. Ten microsatellite markers have been detected from a total of 79 alleles among the 179 buckwheat accessions were collected from Korea. The number of allele per marker locus (N_A) ranged from 2 (GB-FE-001, GB-FE-043 and GB-FE-055) to 31 (GB-FE-035) with an average of 7.9 alleles. GB-FE-035 was the most polymorphic with the highest PIC value 0.93. Major allele frequencies (M_{AF}) for the 10 polymorphic loci varied from 0.12 to 0.97 with a mean allele frequency of 0.57. The expected heterozygosity (H_E) values ranged from 0.05 to 0.94 with an average of 0.53. The observed heterozygosity (H_O) ranged from 0.06 to 0.92 with an average of 0.42. The overall polymorphic information contents (PIC) values ranged from 0.05 to 0.93 with an average of 0.48. The landrace accessions of buckwheat used in the present study were not distinctly grouped according to geographic distribution. The study concludes that the results revealed genetic differentiation was low according to the geographic region because of outcrossing and self-incompatibility. We reported that our analyses on the genetic diversity of common buckwheat cultivars of Korea were performed by using of microsatellite markers.

Key words - Buckwheat (*Fagopyrum esculentum*), Differentiation, Genetic diversity, Microsatellite

Introduction

Common buckwheat (*Fagopyrum esculentum* Moench) is a typical species showing outcrossing and self-incompatiblity belonging to the family Polygonaceae (Park *et al*., 2009; Sharma and Boyes, 1961). Common buckwheat has been widely distributed and a cultivated crop of considerable importance in many countries around the world, in Asia, America and Europe, although the cultivation of this crop has not increased in recent years (Alekseeva, 1986; Kump and Javornik, 1996). The important component of buckwheat seeds are of possession of a well-balanced quantity of essential amino acids and excellent nutritional value (Gao *et al*., 2010; Javomik *et al*., 1981). Besides all, common buckwheat is also an important source as a nectariferous and pharmaceutical plant (Alekseeva, 1986). Most of the varieties of common buckwheat grown are local populations adapted to their environmental conditions through cultivation. For the protection of crop varieties, information on genetic distances among inbreds is important for the identification of essential derivation as well as legal protection of germplasm (Smith *et al*., 1995). Therefore, information about the genetic diversity and population structure in the selection of the breeding material is one of fundamental importance for the improvement of crops (Hallauer and Miranda, 1988). So, the evaluation of germplasm diversity and relationships among the contemporary cultivated and wild varieties and populations is important both for future breeding and for the study of buckwheat evolution (Kump and Javornik, 1996). The genetic diversity among and within common buckwheat cultivars has been studied using allozyme analysis (Ohnishi, 1998) and the origin of cultivated common buckwheat has been studied by the

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diffusion routes analysis using RAPD markers (Murai and Ohnishi, 1996).

Recent advances in molecular biology have offered more suitable molecular markers for assessing genetic diversity than RAPD markers. Among the PCR-based techniques, amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995) and simple sequence repeat (SSR) markers are widely used for studies on genetic diversity of crop species. The advantages of SSR markers are their codominant mode of inheritance and hypervariability, which make them ideal for a wide range of applications (Goldstein and Schlötterer, 1999). Simple sequence repeats (SSRs, also called microsatellites) are abundantly distributed throughout eukaryotic genomes (Litt and Luty, 1989). Microsatellite markers are a powerful tool for the analysis of wide genetic variations within or among populations (Tautz, 1989). In many crops, several recent studies have used SSR markers to assess the genetic diversity, phylogenetic relationships, and population structures of various crops, for example in durum wheat (Thuillet *et al*., 2005), maize (Vigouroux *et al*., 2005), and rice (Li *et al*., 2010). The aims of the present study were to evaluate the genetic diversity, population structure and genetic relationships among geographically diverse accessions of buckwheat landraces of Korea maintaining or conserving in National Agrobiodiversity Center of RDA using SSR markers.

Materials and Methods

Plant materials and DNA extraction

A list of common buckwheat accessions used in this study is given in Table 1. A total of 179 accessions of common buckwheat were obtained from the National Agrobiodiversity Center of the Rural Development Administration (RDA) (http://genebank.rda.go.kr), Korea (GW 19, GG 3, GN 24, JN 14, JB 43, CN 4 and CB 12 accessions). For the DNA extraction, each 5 seeds of 179 accessions were germinated and cultivated in soil trays. Genomic DNA was extracted from green leaves of buckwheat seedling. Total genomic DNA was extracted from the leaves of the seedling using a modified CTAB procedure as previously described by Kump and Javornik (1996). The DNA concentration was determined using a UV−Vis spectrophotometer (ND-1000; NanoDrop, Wilmington, DE, USA). The DNA solution was then diluted to a working concentration with distilled water and stored at -20℃ until use.

Assess of microsatellite markers

All of the SSR markers were obtained from molecular markers of developed by Ma *et al*. (2009) for analysis of genetic diversity and relationships in common buckwheat. Ten polymorphic SSR markers were utilized in a genetic diversity analysis of a common buckwheat population consisting of 179 accessions of diverse regions in Korea (Table 2). The M13F-tail PCR method of Schuelke (2000) was used to measure the size of PCR products (Ma *et al*., 2009). PCR amplification was carried out in a total volume of 20 ul containing 2 ul of genomic DNA (10 ng/ul), 0.2 ul of the specific primer (10 pmol/ul), 0.4 ul of M13 universal primer (10 pmol/ul), 0.6 ul of normal reverse primer, 2.0 ul of 10 x PCR buffer (Takara, Tokyo, Japan), 1.6 ul of dNTP (2.5 mM), and 0.2 ul of Taq polymerase (5 unit/ul; Takara). The reaction mixture was subjected to the following conditions: initial denaturation at 94℃ for 3 min, followed by 30 cycles of denaturation at 94℃ for 30 sec, annealing at 52-55℃ for 45 sec, then 15 cycles at 94℃ for 30 sec, 53℃ for 45 sec, and extension at 72℃ for 45 sec and final extension at 72℃ for 10 min. PCR was carried out in PTC-220 thermocyclers (MJ Research, Waltham, MA, USA). The PCR products were then run on an ABI PRISM 3130xl Genetic Analyzer according to the manufacturer's instructions (Applied Biosystems, USA). Fragments were sized and scored into alleles using GeneMapper v4.0 software (Applied Biosystems, USA).

Data analyses of genetic diversity and population structure

The total number of alleles, allele frequency, gene diversity and polymorphism information content (PIC) per individual SSR locus were calculated with the PowerMarker version 3.25 analysis (Liu and Muse, 2005). Genetic distance between each pair of accessions were calculated from Nei's distance (Nei and Takezaki, 1983) using the program PowerMarker. Nei's distance was calculated and used the unrooted phylogeny reconstruction using neighbor-joining (NJ) method as implemented in PowerMarker version 3.25 (Liu and Muse, 2005). The tree to visualize the phylogenetic distribution of accessions was

Table 1. List of 179 buckwheat accessions of the collection in the RDA.

Sample number	IT or Tem. IT	Region	Country of origin	Sample number	IT or Tem. IT	Region	Country of origin
1	709851	GB	KOR	221	108889	GW	KOR
27	910167	GN	KOR	222	108892	GW	KOR
51	K002646	GN	KOR	223	108934	JB	KOR
53	K002648	GN	KOR	224	108957	GB	KOR
54	K003292	GN	KOR	225	108968	GB	KOR
58	K011766	GW	KOR	226	109053	GB	KOR
141	100906	JN	KOR	228	109078	GB	KOR
142	100973	GB	KOR	229	109095	GB	KOR
144	101006	GB	KOR	230	109106	GB	KOR
145	101022	JB	KOR	233	109175	GB	KOR
146	101091	JB	KOR	237	109601	JN	KOR
147	101120	JB	KOR	238	110977	GB	KOR
148	101271	GW	KOR	239	110978	GB	KOR
149	101282	GW	KOR	241	111123	CN	KOR
150	101389	JB	KOR	244	112812	JB	KOR
151	101391	JB	KOR	247	112911	GG	KOR
153	101431	JB	KOR	249	112949	JB	KOR
154	102359	GB	KOR	250	112957	JB	KOR
155	102780	GB	KOR	252	112982	JB	KOR
157	103026	GB	KOR	254	113033	GB	KOR
158	103069	JB	KOR	255	113051	GB	KOR
159	103093	JB	KOR	256	113066	GB	KOR
160	103119	GN	KOR	258	113083	GB	KOR
163	103569	GN	KOR	260	113086	GB	KOR
165	103633	GN	KOR	261	113087	GB	KOR
167	103710	GN	KOR	262	113088	GB	KOR
169	103836	JB	KOR	263	113123	CB	KOR
170	103881	JB	KOR	264	113126	CB	KOR
173	104133	GB	KOR	266	113200	GB	KOR
174	104139	GB	KOR	268	113250	JB	KOR
175	104236	GB	KOR	269	113266	JB	KOR
177	104328	GB	KOR	270	113276	JB	KOR
178	104429	GN	KOR	271	113296	JB	KOR
179	104461	GW	KOR	272	113306	JB	KOR
181	104526	GW	KOR	274	113347	JB	KOR
182	104551	GW	KOR	275	113353	JB	KOR
183	104769	GN	KOR	276	113358	JB	KOR
187	105304	GW	KOR	277	113371	JB	KOR
190	105398	JB	KOR	278	113392	JB	KOR
194	105473	GB	KOR	279	113406	JB	KOR
198	105523	GB	KOR	280	113413	JB	KOR
200	105543	GB	KOR	282	113458	CN	KOR
207	105856	JB	KOR	283	113577	GB	KOR
210	105954	GN	KOR	284	113582	GB	KOR
212	105997	JN	KOR	285	115174	GB	KOR
214	108713	GB	KOR	286	115180	GB	KOR
215	108752	GB	KOR	287	115186	GB	KOR
218	108786	GB	KOR	293	119935	GB	KOR
219	108852	GW	KOR	294	119936	GB	KOR

Table 1. Continued.

Sample number	IT or Tem. IT	Region	Country of origin	Sample number	IT or Tem. IT	Region	Country of origin
297	134960	GB	KOR	449	185691	GN	KOR
299	134969	GB	KOR	451	185693	GN	KOR
300	134978	GB	KOR	452	185694	GN	KOR
301	135788	GB	KOR	453	185695	GB	KOR
302	136087	GB	KOR	458	185700	GB	KOR
305	138108	GB	KOR	463	185705	JN	KOR
308	138140	GB	KOR	465	185707	JN	KOR
310	138142	GB	KOR	471	185713	JB	KOR
311	138143	GB	KOR	472	185714	JB	KOR
313	138145	GB	KOR	473	185715	JB	KOR
372	148426	GB	KOR	474	185716	JB	KOR
373	148427	GW	KOR	475	185717	CN	KOR
374	148428	GB	KOR	477	185719	CB	KOR
375	148429	CB	KOR	478	185720	CB	KOR
377	155169	GB	KOR	480	185722	CB	KOR
378	158263	GW	KOR	481	185723	CB	KOR
380	160614	JN	KOR	482	185724	CB	KOR
387	162837	CB	KOR	495	191108	GN	KOR
389	162883	JB	KOR	498	191639	GW	KOR
390	162884	JB	KOR	499	194510	GN	KOR
392	175826	GB	KOR	500	194511	GN	KOR
394	175860	GB	KOR	502	194513	JN	KOR
395	175869	GB	KOR	503	194514	JB	KOR
403	176005	GG	KOR	506	195499	GW	KOR
404	178414	JB	KOR	507	195500	GW	KOR
405	178415	CN	KOR	536	208546	GB	KOR
406	178416	CB	KOR	538	208548	GB	KOR
407	178417	JB	KOR	544	208554	JN	KOR
421	180529	JB	KOR	545	208555	JN	KOR
422	180606	JB	KOR	548	208826	JN	KOR
423	180612	GN	KOR	549	208852	GW	KOR
424	180619	GN	KOR	552	209882	GN	KOR
425	180643	GN	KOR	555	209885	GN	KOR
432	180927	CB	KOR	556	210197	GW	KOR
433	180928	CB	KOR	557	210198	GW	KOR
436	180931	JB	KOR	561	212210	JN	KOR
437	181904	JB	KOR	562	212211	JN	KOR
441	181973	JB	KOR	563	212212	JN	KOR
445	185687	GG	KOR	564	212213	JN	KOR
446	185688	GN	KOR	567	214694	GW	KOR
448	185690	GN	KOR				

[†]CB, Chungbuk; CN, Chungnam; GB, Gyeongbuk; GG, Gyeonggi; GN, Gyeongnam ; GW, Gangwon; JB,Jeogbuk; JN, Jeonnam.

constructed using the software MEGA version 5.03 (Tamura *et al*., 2007) embedded in PowerMarker. The model-based program STRUCTURE (Pritchard *et al*., 2007) was utilized to infer population structure and assign individuals to populations based on the SSR genotypes using a burn-in of 50,000, run length of 100,000 and a model allowing for admixture and correlated allele frequencies. The number of populations (K) was set from 1 to 10, with 3 independent runs each. The most probable value (K) corresponds to the peak in the D(K), which is an *ad hoc* statistic D(K), assisted with L(K), L'(K)

and L"(K) (Evanno *et al*., 2005). The D(K) perceives the rate of change in log probability of the data with respect to the number of groups inferred by STRUCTURE.

Results and Discussion

Profile of microsatellite markers

We assessed the genetic variability of common buckwheat landrace accessions representing diverse regional collections in Korea using SSR markers (Table 1). Ten microsatellite markers detected a total of 79 alleles among the 179 buckwheat accessions (Table 3). The number of allele per SSR marker locus (N_A) ranged from 2 (GB-FE-001, GB-FE-043 and GB-FE-055) to 31 (GB-FE-035) with an average of 7.9 alleles. The GB-FE-035 marker produced 31 alleles that were the highest number of alleles of markers and the highest PIC value was 0.93. The major allele frequencies (M_{AF}) for the 10 polymorphic loci varied from 0.12 (GB-FE-035) to 0.97 (GB-FE-169) with an average allele frequency of 0.57. The expected heterozygosity (H_E) values ranged from 0.05 to 0.94 with an average of 0.53 and the observed heterozygosity (H_O) ranged from 0.06 to 0.92 with an average of 0.42. The overall polymorphic information contents (PIC) values ranged from 0.05 to 0.93 with an average of 0.48. We could be confirmed the genetic diversity among 179 common buckwheat accessions in this study. These results are compared with that of detected in buckwheat using SSR markers by Iwata *et al*.(2005). Our results indicated that the average H_E value was lower than that of the 19 cultivars (0.819) used by Iwata *et al*.(2005) in buckwheat. It is inferred that the value was relatively low in our study, because in common buckwheat analyzed genetic diversity was based on Korean indigenous resources, which were collected in Korea.

Genetic diversity and phylogenetic relationships

A neighbor-joining tree of 179 landraces accessions was constructed based on Nei's genetic distance. The genetic distance matrix generated by PowerMarker software and

Table 2. List of microsatellite markers used in this study.

Marker	GenBank accession	Primer sequence (5'-3')	Repeat Motif
GB-FE-001	EU998635	F-TGAAACCCAACCATCAGG R-CGACAGTGGCTGGAGAAC	(CAA)7
GB-FE-012	EU998636	F-ACTGCACCCCAGAGGATT R-GCTGTATCCATGCCCGTA	(CAG)5(CT)(CAG)&(GAK)8
GB-FE-014	EU998637	F-AGGAGCAGAGGTGGTGGT R-CGGAGCCTCTGCAACC	(GA)10C(GA)
GB-FE-035	EU998638	F-TGCAATGACTTGGAGGAGA R-ACCACCATTCAACAAGCG	(GAY)14(GGT)(GAB)41
GB-FE-043	EU998639	F-TTCAGCACCTGGATGGAC R-TGTCCCCAATGTGAAAGG	(CCA)5
GB-FE-054	EU998640	F-TGTTGGACTTCCTAGACCTG R-CATGAAAAGGGGATGCAA	(TR)12
GB-FE-055	EU998641	F-CTGCTTGGATCCCATTGA R-AGCCTCTCGATCCCTCTG	(GAK)6&(GAT)3&(GAT)2
GB-FE-080	EU998642	F-CGAGGTGGGCAGTAGAGA R-GAGGAGGACGAGGAGGTG	(CST)7
GB-FE-169	EU998643	F-CAACCCTATGCAGCGTTC R-GAGGGGAAGCTGCTTGTT	(ACA)6
GB-FE-191	EU998644	F-AGT AATCAATGACCAGCACGC R-CTGATGGAGGATGCCAAA	(CAT)5

used to construct an unrooted neighbor-joining tree. The dendrogram revealed a complex accession distribution pattern (Fig. 1A). DNA polymorphism detected by 10 SSR markers allowed genetic distance estimation and the UPGMA tree showed that 179 accessions of Korea buckwheat cultivars were classified in three major groups. The genetic distance among the buckwheat populations from 8 different regions was also used to construct an UPGMA tree (Fig. 1B). The genotypic diversity of buckwheat from 8 geographical regions is compared in Table 4. The genetic diversity of buckwheat populations from 8 geographical regions was characterized by an average of 4.08 alleles, ranging from 2 in GG to 6.0 in GB province. The mean frequency of major alleles (M_{AF}) per locus was 0.613, varying from 0.542 in CB to 0.792 in GG province. The expected heterozygosity (H_E) values ranged from 0.310 (GG) to 0.549 (CB) with an average of 0.481 and

Table 3. Characterization of the 10 microsatellite loci among common buckwheat base on 179 collected germplasm accessions.

Marker	M_{AF}	N_A	H_E	H_O	PIC
GB-FE-001	0.61	2.00	0.48	0.55	0.36
GB-FE-012	0.63	8.00	0.55	0.44	0.51
GB-FE-014	0.47	5.00	0.67	0.60	0.62
GB-FE-035	0.12	31.00	0.94	0.25	0.93
GB-FE-043	0.77	2.00	0.35	0.22	0.29
GB-FE-054	0.37	9.00	0.74	0.14	0.70
GB-FE-055	0.52	2.00	0.50	0.92	0.37
GB-FE-080	0.78	6.00	0.36	0.36	0.33
GB-FE-169	0.97	5.00	0.05	0.06	0.05
GB-FE-191	0.42	9.00	0.67	0.62	0.61
Total	5.67	79.00	5.31	4.15	4.78
Mean	0.57	7.90	0.53	0.42	0.48

M_{AF}, major allele frequency; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphic information content.

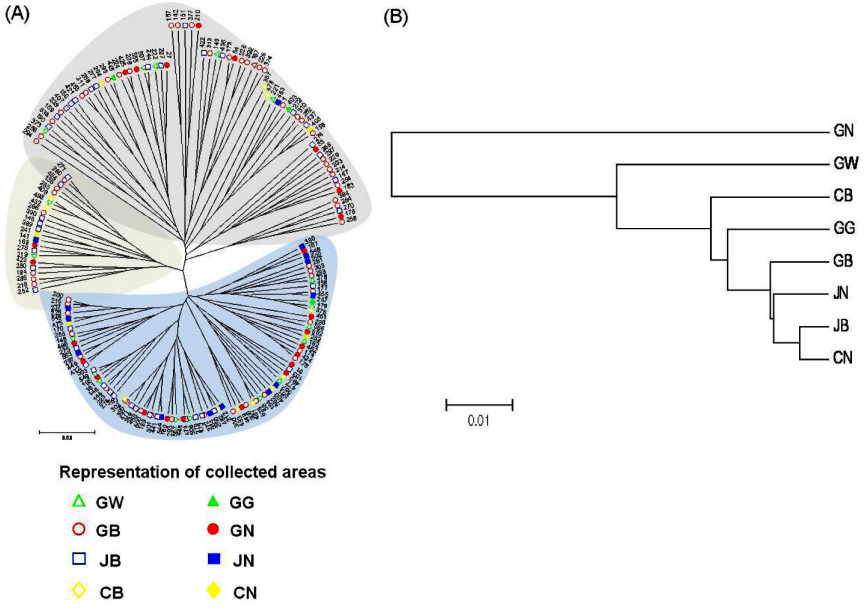

Fig. 1. Unrooted neighbor-joining trees of 179 buckwheat accessions collected from different regions in Korea based on Nei's genetic distances among 10 SSR loci (A) and the genetic relationships among different populations in different regions (B).

the observed heterozygosity (H_O) ranged from 0.325 (CN) to 0.521 (CB) with an average of 0.415. The overall polymorphic information contents (PIC) values ranged from 0.261 (GG) to 0.497 (CB) with an average of 0.428.

The phylogenetic distribution of buckwheat accessions and populations from the 8 geographical regions indicated the complexity in distribution and did not clustering from the same regions. This result suggests that common buckwheat widely dispersed with small local differentiation due to strong migration pressure into new geographical regions. Similar results were reported by other studies (Cho *et al*., 2011; Kump and Javornik, 1966).

Population structure

In order to check the subdivision, a model-based clustering method for multi-loci genotype data was performed to determine the population structure and assign individuals to populations using STRUCTURE. The most probable structure number of K was calculated based on Evanno *et al*. (2005) using and *ad hoc* statistic D(K), assisted with L(K), L'(K) and L"(K). The highest value of D(K) for the 179 buckwheat accessions was K = 2 (Fig. 2A). The model-based structure analysis revealed the presence of two subpopulations (Fig. 2B). As shown in Table 5, most of the 179 buckwheat accessions, 114 (63.7%) accessions were classified into one of the two genetic groups, whereas 65 (36.3%) of the entire accessions were classified as admixed forms with varying levels of membership shared among the two genetic groups (Fig. 2B and Table 5). Group 1 consisted of 57 accessions, involving 7 GW, 10 GN, 20 GB, 2 JN, 13 JB, 1 CN and 4 CB accessions. Group 2 (G2) consisted of 57 accessions, including 6 GW, 1 GG, 5 GN, 22 GB, 5 JN, 13 JB, 2 CN and 3 CB

Table 4. Characterization of the 10 microsatellite loci according to 8 geographical regions in Korea.

Regions	Sample Size	M_{AF}	N_A	H_E	H_O	PIC
GW	19	0.586	3.80	0.517	0.408	0.459
GG	3	0.792	2.00	0.310	0.383	0.261
GN	24	0.609	4.50	0.491	0.448	0.438
GB	60	0.588	6.00	0.502	0.401	0.450
JN	14	0.580	3.90	0.515	0.449	0.454
JB	43	0.558	5.70	0.540	0.389	0.483
CN	4	0.650	2.70	0.422	0.325	0.378
CB	12	0.542	4.00	0.549	0.521	0.497
Total	179	4.905	32.60	3.846	3.324	3.420
Average		0.613	4.08	0.481	0.415	0.428

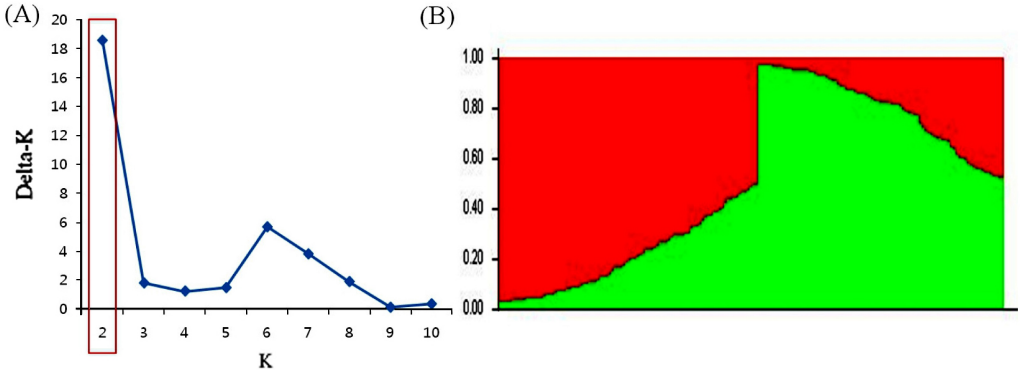

Fig. 2. Population structure of 179 buckwheat accessions based on 10 SSRs (K=2). (A), Estimation of the number of populations for K ranging from 1 to 10 by calculating delta K values. Delta-K analysis of LnP(D), according to Evanno *et al*. (2005). (B), Model-based clustering for each of the 179 accessions examined based on the 10 SSR markers using STRUCTURE.

Table 5. Distribution (inferred) of accessions from different regions to each clusters and admixture.

Region	Cluster 1	Cluster 2	Admixture	Total
GW	7	6	6	19
GG	0	1	2	3
GN	10	5	9	24
GB	20	22	18	60
JN	2	5	7	14
JB	13	13	17	43
CN	1	2	1	4
CB	4	3	5	12
Total	57	57	65	179

accessions. The result indicated that the 179 landrace accessions of buckwheat were not distinctly grouped according to geographic distribution.

In this study, the genetic diversity of common buckwheat accessions was studied based on microsatellite markers in order to provide useful information for conservation and utilization of buckwheat genetic resources in Korea. The genetic diversity, phylogenetic relationships and population structure of the common buckwheat landraces in Korea were analyzed by the statistics methods. The results shown that there are genotypic variations exists in common buckwheat accessions collected from 8 different regions in Korea. However, the present study showed that UPGMA tree and the division of genetic structure do not match between the model-based genetic structure and the geographical regions. In addition, the genotypes collected from the same geographical places did not form a single cluster or grouping. Similar observations were made by Masud *et al*.(1995) in pumpkin. The average number of alleles per locus among the 179 accessions of the RDA genotyped by 10 SSR markers was 7.9, which is slightly lesser than that of the population studied by Iwata *et al*. (2005). Konishi *et al*. (2006) reported an average SSR PIC value of 0.79 among a worldwide core collection of common buckwheat accession and the PIC value obtained from our analysis were 0.48. Although common buckwheat in Korea, in this study shows moderate levels of genetic diversity, the parameters are lower than the expected from outcrossing and 8 areas in Korea. This genetic variability is highly dependent on the number of samples and on the areas from which the samples were collected. Sinha *et al*. (1991) reported that selection of parents from distantly placed clusters exhibited significant high heterotic segregants and the decline of cultivated areas may be a major factor.

In conclusion, the results suggested that genetic differentiation was relatively low according to the geographic regions because of the characters of outcrossing and self-incompatibility. Moreover, these reasons could be explained by various factors, such as migration into new geographical areas and adaptation to the climate of Korea. Murai and Ohnishi (1996) had noted a gradual decline of polymorphism with the migration from the center of origin place of the species (Yunnan or Sichchuan province). Ohnishi (1993) describes common buckwheat as a widely dispersed crop with small local differentiation. These results, including the genotype-specific alleles, genetic diversity, and population structure information, will facilitate the use of the buckwheat germplasm for crop improvement. Evaluations of genetic diversity of Korea landraces have played an important role in the conservation program of plant genetic resources. This diversity information based on genetic variation may contribute to the evaluation of other germplasm collections and genetic analysis of common buckwheat species to elucidate their evolutionary and phylogenetic relationships and to broaden the genetic base of modern buckwheat cultivars.

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