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Studies on Genetic Diversity of Buckwheat Germplasms

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Abstract - Many research results have indicated that many kinds of useful ingredients are rich in buckwheat, which have high nutritional values and medicinal properties, so, buckwheat has been cultivated around Asia and Europe. In this paper, genetic diversity of common and tartary buckwheat germplasms were studied based on morphological and molecular markers in order to provide useful information for conservation and utilization of buckwheat genetic resources. The length, width, thick, value and weight of the seed of the common and tartary buckwheat were measured and analyzed by the statistics methods. The result has shown that there are morphological variation both in common buckwheat and tartary buckwheat seeds collected from different regions. It also has shown that the morphological variation of tartary seeds was significantly correlated to geographical regions. The nuclear ribosomal internal transcribed spacers (ITS) of the tartary buckwheat collected from different countries were cloned, sequenced and statistically analyzed. The data showed that ITS sequences were informative to analyze the phylogeny of the tartary buckwheat and the data also showed that the genetic distances varied among different tartary buckwheat seeds collected from different countries.

Key words - Common buckwheat, Tartary buckwheat, morphological characterization, ITS, genetic diversity

Abbreviations - SL : Seed Length, SW : Seed Width, ST : Seed Thick, SV : Seed Value, SW/SL : Seed width ÷ Seed Length

Introduction

Buckwheat belongs to *Fagopyrum* Mill. (*polygonaceae*) and distributes in Asia and Europe extensively. In general, only two species of buckwheat, namely common buckwheat and tartary buckwheat are cultivated species. Common buckwheat is a self-incompatible species. The main producers of common buckwheat are China, Russian Federation, Ukraine and Kazakhstan (Campbell, 1995). Major exporters are China, Brazil, France, USA and Canada. Japan accounts for almost all of the world's buckwheat imports. The other cultivated species, Tartary buckwheat, is a self-pollination species. It is also produced in many areas of the world but generally is consumed or traded locally. *F. tataricum,* because of its frost tolerance potential, is mainly cultivated in the high altitude mountainous areas of Asia and to a lesser extent elsewhere. In many areas the trend is for replacement of common buckwheat, which has lower yielding ability and lacks frost tolerance, with other crops (Bisht *et al.*, 2006, 2007). At these altitudes Tartary buckwheat frequently yields out common buckwheat. Therefore, in some areas of India, Nepal and China common buckwheat production is declining but Tartary buckwheat is remaining stable or increasing.

Buckwheat grain is grown mainly for human consumption and as animal feed, although it can also be used as a green vegetable, a green manure cover crop and a smother crop to crowd-out weed and as a source of buckwheat honey. Many kinds of nutritional ingredients are rich in buckwheat. Flavones, flavonoids, sterols, fagopyrin, and thiamin binding proteins in buckwheat seeds possess potential effects in treating some chronic diseases. In buckwheat, the main component of flavonoids (80%) is rutin. Rutin can reduce high blood pressure, decrease the permeability of the blood vessels (Abeywadena *et al.*, 2001; Schilcher *et al.,* 1990), reduce the risk of arterioselerosis (Wojcicki *et al.*, 1995), antagonize the increase of capillary fragility associated with haemorrhagic disease,

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and have an antioedema effect and antioxidant activity (Holasova *et al.*, 2001; Park *et al*., 2000).

The present study was undertaken with the objectives of estimating genetic diversity in buckwheat in order to provide useful information for conservation and use of buckwheat genetic resources. There were two ways to study genetic diversity of buckwheat, they are morphological and molecular markers. In morphologic study, the seeds of common and tarary buckwheat from different collection regions were involved. The seed length, seed width, seed thick and weight, were measured, meanwhile, the seed value and ratio of seed width to length also were computed. The data of morphological descriptors of seeds was subject to statistic analyses, which involved analysis of variance and classification. In the molecular study, sequences of the internal transcribed spacers (ITSs) of nuclear ribosomal DNA (nrDNA) were analyzed on common and tartary buckwheat. The internal transcribed spacers (ITS) are non-coding nrDNA sequences occurring in eukaryotes, which are located between the 18s and 5.8s coding regions (ITS1) and between the 5.8s and 26s coding regions (ITS2). While the sequences of the coding regions are highly conserved, the ITS-regions are more variable and have proven to be useful for phylogenetic analyses. In this work, 7 populations of tartary buckwheat were studied in length variation, GC content and analysis of homology similarity.

Materials and Methods

Plant material

Seeds of common buckwheat and tartary buckwheat were studied. The seeds of common buckwheat were collected from different populations in Korea; the seeds of tartary buckwheat were collected from different countries and regions in Europe and Asia. 35 assembles of tartary buckwheat and 31 assembles of common buckwheat were studied in this paper, some details were involved in Table 1.

Scoring of morphological descriptors

Ten seeds were randomly selected from different population of common buckwheat and tartary buckwheat, respectively. Each seed, there were six descriptors to be measured and computed. Seed length (SL), seed width (SW) and seed thick (ST) were measured by micrometer calipers (0.01 cm); single seed weight were measured by electronic balance (0.1 mg). The seed value was computed in accordance with the formula (SL x SW x ST), which was a relative concept and the ratio of SW to SL also was computed. The greatest value of seed length, seed width and seed thick were recorded in measure process. The data measured from seeds was analyzed by the SPSS 11.5, analysis of variance (ANOVA) was carried out and Classification (cluster analysis) was also performed.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from fresh leaves using SDS extraction protocol (Sambrook *et al.,* 1989). To detect the complete sequence of the ITS region, primer set of ITS5 and ITS4 were used for amplification by PCR. PCR amplification was carried out in a volume of 25 μl containing 10 ng of DNA template, 1 μl of each primer, 2 mM of each dNTP, 2.5 mM $MgCl_2$, and 0.2 μl of Ex *Taq* DNA polymerase. Amplification was conducted in a Peltier Thermal Cycler (PC808, ASTC, Japan). PCR cycles were as follows: one cycle of 4 min at 94℃, 35 cycles of 1 min at 94℃, 1 min at 48℃, and 1.5 min at 72℃, followed by 1 cycles of 7 min at 72℃, with a final extension step at 4℃. The PCR products were separated using 1.0% agarose gel electrophoresis. For sequencing, we used the same primer sets as for the PCR amplification. Sequences were aligned manually. Nucleotide additives observed on the electrophotogram of the direct sequence were coded according to the IUPAC (IUB) code. The ITS4 and ITS5 primers were 5-GGAAGTAAAAGTCGTAACAAGG-3 and 5-TCCTCCGCTTATTGAT ATG C-3.

ITS data analyses

All PCR product purification used QIAquick PCR Purification kit and DNA sequencing was done by the fluorescent dye terminators method. Sequence analysis was done based on ITS sequences of all species by DNAMAN Version 5.2.9 software, to verify the two internal transcribed spacer regions (ITS1 and ITS2) and a coding region (5.8s) in nuclear ribosomal DNA.

Table 1. List of buckwheat used for morphological characterization

Species	Serial	Accession no.	Collection region	Type
Tartary Buckwheat	K1	KW1	Lasa Central Tibet, China	Wild
	K4	KW4	Markan EasternTibet ,China	Wild
	K6	KW6	Ltanga Sichuan China	Wild
	K7	KW7	Lixian Sichuan, China	Wild
	K9	KW9	Northern Pakistan, Pakistan	Cultivate
	K10	KW10	unknown Kashmir, India	Cultivate
	K11	KW11	unknown Kashmir, India	Cultivate
	K15	KW15	Ghandruk, Central Nepal	Cultivate
	K16	KW16	Chinbu, Central Nepal	Cultivate
	K17	KW17	Rara, Western Nepal	Cultivate
	K18	KW18	Gesta, Bhutan	Cultivate
	K19	KW19	Thimipu, Bhutan	Cultivate
	K20	KW20	Chumey, Bhutan	Cultivate
	K21	KW21	Unknown, Luxemburg	Cultivate
	K23	KW23	Sevnica, Slovenia	Cultivate
	K26	KW26	Xianwei Ynnan, China	Cultivate
	K27	KW27	Quifing Ynnan, China	Cultivate
	K28	KW28	Eryuan Ynnan, China	Cultivate
	K29	KW29	Shilin Ynnan, China	Cultivate
	K30	KW30	Fuming Ynnan, China	Cultivate
	K32	KW32	Simao Yunnan, China	Cultivate
	K34	KW34	Yanmen Sichuan, China	Cultivate
	K35	KW35	Guanluo Sichuan, China	Cultivate
	K36	KW36	Zhajial Sichuan, China	Cultivate
	K38	KW38	Zhongza Sichuan, China	Cultivate
	K39	KW39	Yulin, China	Cultivate
	K41	KW41	Xinning, China	Cultivate
	K42	KW42	Ynnan Yunnan, China	Cultivate
	K43	KW43	Ynnan Yuannan, China	Cultivate
	K45	KW45	Unknown, Japan	Cultivate
	K46	KW46	Unknown, Nepal	Cultivate
	K47	KW47	Unknown, Nepal	Cultivate
	K48	KW48	Unknown, Nepal	Cultivate
	K49	KW49	Unknown, Nepal	Cultivate
	K50	KW50	Unknown, Nepal	Cultivate

Table 1. List of buckwheat used for morphological characterization (Continued)

Species	Serial	Accession no.	Collection region	Type
Common Buckwheat	C1	'96 AB − 44 KLB 75035	Korea	Cultivate
	C2	141450 KLB 86007	Korea	Cultivate
	C3	'96 AB − 45 KLB 86003	Korea	Cultivate
	C4	'96 AB − 46 KLB 86004	Korea	Cultivate
	C5	'96 AB − 49 617	Korea	Cultivate
	C6	148398 75018	Korea	Cultivate
	C7	'96 AB − 06 705	Korea	Cultivate
	C8	141451 KLB 86008	Korea	Cultivate
	C9	148387 75004	Korea	Cultivate
	C10	'96 AB − 10 KLB 75039	Korea	Cultivate
	C11	'96 AB − 37 526	Korea	Cultivate
	C12	148388 75005	Korea	Cultivate
	C13	'96 AB − 14 KLB 86006	Korea	Cultivate
	C14	'96 AB − 45 785	Korea	Cultivate
	C15	138143	Korea	Cultivate
	C16	'96 AB − 18 KLB 8616	Korea	Cultivate
	C17	'96 AA − 12 KIVB 95028	Korea	Cultivate
	C18	148397 75017	Korea	Cultivate
	C19	'96 AB − 46 766	Korea	Cultivate
	C20	'96 AB −23 KIVB 94083 − S1	Korea	Cultivate
	C21	148395 75014	Korea	Cultivate
	C22	'96 AB − 24 KLB 94084	Korea	Cultivate
	C23	148411 75031	Korea	Cultivate
	C24	'96 AB − 18 1033	Korea	Cultivate
	C25	'96 AB − 16 690	Korea	Cultivate
	C26	'96 AB − 07 609	Korea	Cultivate
	C27	'96 AB − 44 KLB 75035	Korea	Cultivate
	C28	148424	Korea	Cultivate
	C29	148389 75006	Korea	Cultivate
	C30	148391 75009	Korea	Cultivate
	C31	141456 KLB 86013	Korea	Cultivate

Results

The morphological descriptors of common buckwheat and tartary buckwheat were measured and computed, respectively. In order to consider these differences clearly, all morphological data from common buckwheat and tartary buckwheat as a whole were carried out variance analysis, respectively, and the results were showed in Table 2. There was significant morphological variance of seeds both in common buckwheat and tartary buckwheat. To common buckwheat, the variance source of SW, ST, SV and Weight, came from populations was above 50%, while, that of SL and SW / SL was below 50%. To tartary buckwheat, the variance source of

Table 2. Variance analysis of seed descriptors in buckwheat

Species	Variance source	SL	SW	ST	SV	Weight	SW / SL
Common Buckwheat	Between Groups	0.323	0.519	0.546	0.563	0.786	0.452
	Within Groups	0.677	0.481	0.454	0.473	0.214	0.548
	F	4.429**	10.047**	11.202**	11.989**	34.205**	7.682**
Tartary Buckwheat	Between Groups	0.650	0.760	0.600	0.701	0.758	0.693
	Within Groups	0.350	0.240	0.400	0.299	0.242	0.307
	F	16.216**	26.974**	13.014**	20.413**	27.334**	19.630**

Significant level: significant (*), P<0.05, very significant(**), (P<0.01).

Table 3. Mean value and coefficient of variation of descriptors in buckwheat

Species	Character	Mean	Std. Deviation	Minimum	Maximum	Coefficient of Variance
Common Buckwheat	SL	6.29	0.56	4.54	7.90	0.090
	SW	4.32	0.93	2.95	9.00	0.215
	ST	3.78	0.58	2.60	6.45	0.154
	Weight	26.19	3.69	15.70	35.50	0.141
	SW / SL	1.52	0.29	0.61	2.145	0.191
	SV	104.95	39.19	38.70	318.28	0.373
Tartary Buckwheat	SL	4.86	0.63	2.75	6.40	0.130
	SW	3.21	0.59	2.00	5.70	0.183
	ST	2.75	0.35	1.65	3.95	0.127
	Weight	16.23	5.46	3.40	30.74	0.313
	SW / SL	0.67	0.17	0.40	1.63	0.254
	SV	43	12.34	18.21	88.43	0.297

SL: Seed Length, SW: Seed Width, ST: Seed Thick, SV: Seed Value, Weight: Weight of single seed, SW/SL: Seed width ÷ Seed Length.

all morphological descriptors, came from populations were above 60%. To tartary buckwheat, variance source mainly come from different populations. So, geographical distribution plays a more important role in morphological variation to tartary buckwheat than that of common buckwheat. To common buckwheat and tartary buckwheat, all seeds from different populations as a whole, the mean of six morphological descriptors have been computed, and the value of coefficient of variance in different descriptors was compared, the results were shown in Table 3. Compared common buckwheat with tartary buckwheat in Table 3, each mean value from common buckwheat is higher than that of tartary buckwheat. The coefficient of variance (CV) can show the degree of variance for different morphological descriptors in same population. To common buckwheat, the CV value of SV is highest and SL is lowest, while, to tartary buckwheat, the CV value of weight is highest and ST is lowest. Two kinds of buckwheat showed different traits of morphological variance.

The mean of SV of seeds and the mean of SW / SV from different population in common buckwheat and tartary buckwheat showed the all mean value of SV of common buckwheat nearly are larger than that of tartary buckwheat and tarary buckwheat seed shape are conical, mostly. To common buckwheat, with the increasing of SW / SL, the seed shape will change from conical to triangular. Some photos of seeds were shown in Fig. 1.

In order to study on relationship of morphological variance among populations in common buckwheat and tartary buckwheat, Q-cluster analysis was carried out based on morphological characters. Different populations were divided

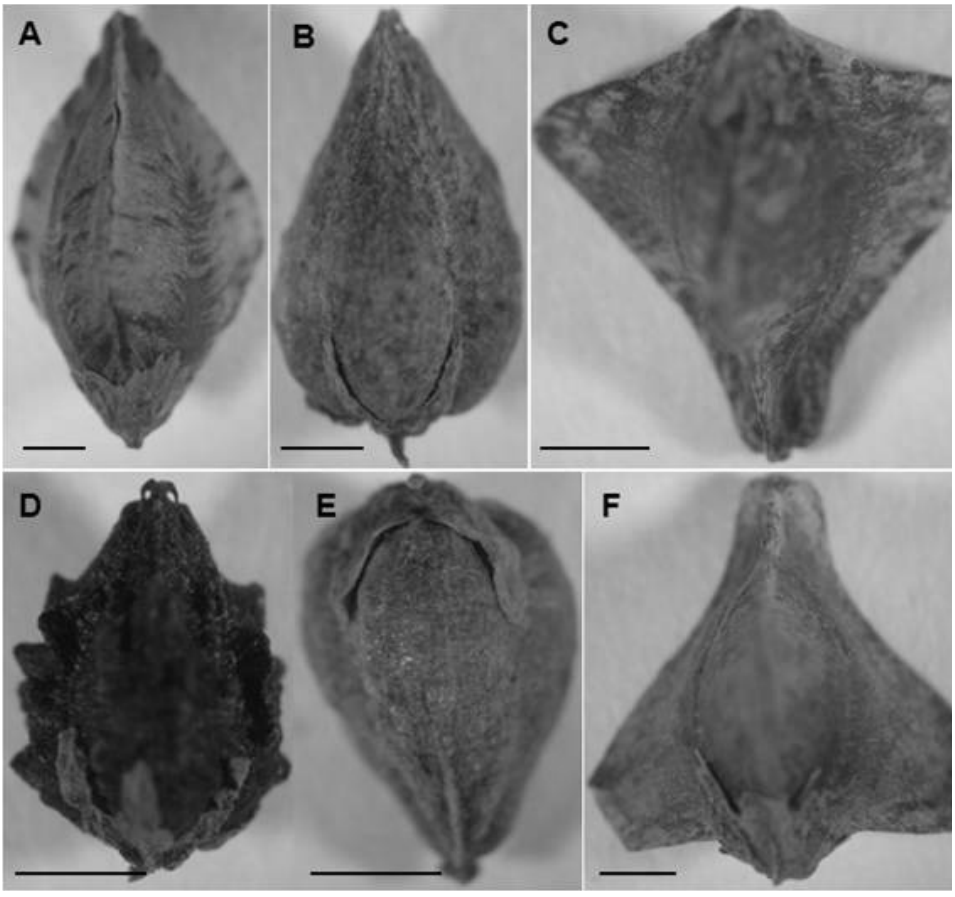

Fig. 1. The photos of seeds of common buckwheat and tartary buckwheat.
A: Seed from C30. B: Seed from K11. C: Seed from C31. D: Seed from K29. E: Seed is from K17. F: Seed from C26.

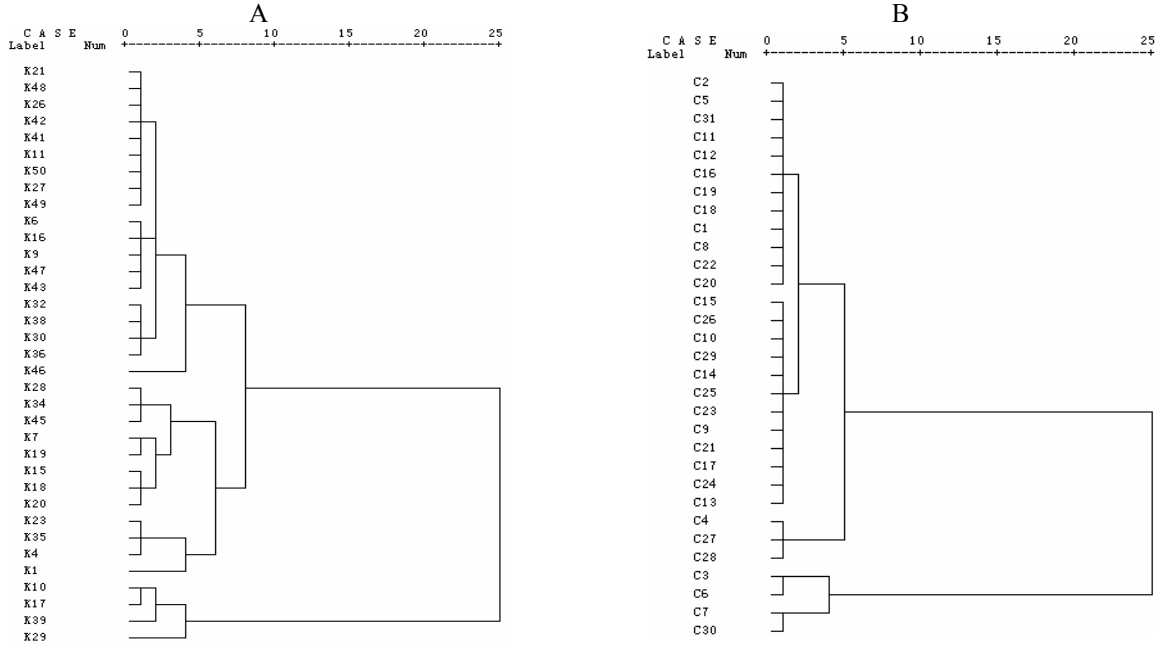

Fig. 2. Q cluster analysis of buckwheat populations.
A. 35 tartary buckwheat populations B. 31 common buckwheat populations

into two clades (I, II) .Most populations were assembled in clade I, and there were only four populations involved in clade II, respectively. There was relative close genetic distance among different populations. The results were shown in Fig. 2. The populations of tartary buckwheat also were divided into clade I and clade II. However, clade I was divided into three subgroups (A, B, C), the situation of cluster analysis is more complicated than common buckwheat. In subgroup A, it involved nearly all Nepal populations, except for K15 and K17; K11, an India population, K9, a Pakistan population also were involved ; Subgroup B, it involved all Bhutan population, they were K18, K19, K20, it also involved one Japan population, K45; K23, a Slovenia population, and K1 and K4 two wild population, collected from China Tibet, were involved in subgroup C. To clade II, only 4 populations were involved, that came from India K10, Nepal K17, K29 and K39, come from China. The other populations collected from China in different regions were involved in two clades. Compared with common buckwheat, tartary buckwheat has more morphological variation among different populations. The Fig. 2-B can indicate that the morphological variation of tartary buckwheat had a certain relationship with geographical distribution.

In this experiment, 9 individuals were involved from 9 common buckwheat populations, they were C1, C3, C4, C6, C9, C11, C13, C14 and C20; 7 tartary buckwheat individuals were involved from 7 populations, they were K9, K10, K21, K27, K30 and K35. Considered the results about the morphological variation of six descriptors as mentioned above that there were more obvious morphological variation among different populations in tartary buckwheat, compared with common buckwheat, in order to discuss the relationship between morphological variation and molecular variation in different populations, so tartary buckwheat finally was decided to be subject to ITS experiment, in order to determine whether there is genotype variation in the ITS region among different populations.

The PCR products were sent to bio-company to be cloned and sequencing. Sequence analysis was done based on ITS sequences of all species by DNAMAN Version 5.2.9 software, to verify the two internal transcribed spacer regions (ITS1 and ITS2) and a coding region (5.8s) in nuclear ribosomal DNA. The multiple alignment was carried out, the genotype varieties occurred mainly in ITS1 and ITS2 region. There were slight length differences of ITS, ITS1 and ITS2 among 7 individuals, the longest length of ITS1 is 600 bp, and the shortest is 598 bp; the longest ITS2 Length is 223 bp and the shortest is 221 bp. There was no difference in 5.8s region. The GC content is stable in ITS1, ITS2 and 5.8s region. There is no huge length variation and GC content among 7 individuals. Meanwhile, the Length and GC content in ITS, ITS1, ITS2 and 5.8s regions were shown in the Table 4.

In order to analyze the homology similarity among 7 individuals, the homology tree of 7 individuals was constructed. The homology similarity of 7 sequences is very high, with above 98%. K9, collected from Pakistan, and K35, collected from Guanluo Sichuan, in China, were divided into one group, they have 99% similarity; K10, collected from India, was together with K27, K32 and K30, which collected from Yunnan and Sichuan, in China, especially, homology similarity of

Table 4. Length variation and GC content of the ITS region

Lines	*K9*	*K10*	*K21*	*K27*	*K30*	*K32*	*K35*
ITS Length	599 bp	600 bp	598 bp	599 bp	559 bp	599 bp	559bp
(G+C)%	68.7	68.6	68.0	68.8	68.5	68.8	68.7
ITS1 Length	213 bp	213 bp	213 bp	213 bp	213 bp	213 bp	213bp
(G+C)%	71.4	71.5	71.2	71.8	71.4	71.8	71.3
5.8s Length	164 bp	164 bp	164 bp	164 bp	164 bp	164 bp	164bp
(G+C)%	57.8	58.0	57.6	58.0	57.8	58.0	57.9
ITS2 Length	222 bp	223 bp	221 bp	222 bp	222 bp	222 bp	222bp
(G+C)%	75.6	74.7	73.5	73.8	74.7	73.8	75.1

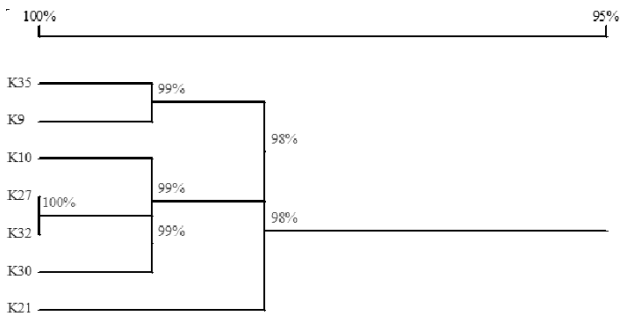

Fig. 3. Homology tree of 7 tartary buckwheat sequences.

K27 and K32 is 100%; only K21, collected from Luxemburg, in Europe, was relatively lower homology similarity with other 6 sequences. Except K21, the other 6 samples collected in different regions in Asia, which were very close distance in geographical distribution, and they represent rather high homology similarity among them, compared with K21, so a conclusion may be derived that the homology similarity has a certain correlation relationship with geographical distribution (Fig. 3).

Discussion

Common buckwheat has been cultivated for centuries in the world, it has longer cultivate history than tartary buckwheat, in order to meet the need of food and commerce. These variety with good agronomic traits, such as high yield, will be selected to be cultivated. In this paper, all the common buckwheat are cultivated variety, they may have been selected for farmers. So though they were collected from different regions in Korea, there was no significant morphological variation of seeds. In another word, a factor of human selection may play a role in affecting the morphological variation of common buckwheat. Meanwhile, natural environment factor may also play a role in affecting the morphological variation of common buckwheat. In this paper, all the common buckwheat seeds are collected in Korea, the growing environment is similar. While, tartary buckwheat were collected in different countries and regions, they grow in different natural environment, So, this is one reason why morphological variation source from different populations in common buckwheat is less than that of tartary buckwheat.

In this paper, only tartary buckwheat individuals were carried out the ITS analysis. In the Q cluster analysis for the morphological variation, the population K9, K21, K27, K30, K32 and K35 were involved in the subgroup A and subgroup C in clade I, and K10 was involved in clade II. They represented different morphological variation, 7 tartary buckwheat individuals represented rather high homology similarity of genotype. There is no significant relationship between morphological variation and genotype variation in ITS region. The gene of morphological phenotype may not be involved in ITS region, which may be one reason to interpret this uncorrelation. Some papers showed that tartary buckwheat is self-pollinating crop, which lead to the possibility of introducing other genetic materials is little, so, tartary buckwheat maybe evolve slowly. This also may be one reason that why there was high homology similarity among 7 tartary buckwheat individuals.

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