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Assessment of genetic diversity and relationships among *Coix lacryma-jobi* accessions using microsatellite markers

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Abstract

The present study describes the assessment of genetic diversity and relationships among 79 Job's tears (*Coix lacryma-jobi* L.) accessions collected from China and Korea using 17 microsatellite markers. A total of 57 alleles were detected with an average of 3.4 alleles per locus. A high frequency of rare alleles (36.3 %) was observed within the collection. Values for observed (H_O), expected heterozygosity (H_E) and Shannon's information index (I) within the analysis ranged from 0.00 (GBssrJT183) to 0.81 (GBssrJT130), from 0.01 (GBssrJT170) to 0.65 (GBssrJT130) and from 0.034 (GBssrJt170) to 1.13 (GBssrJT130), respectively. The locus GBJT130 was the most informative marker with the highest values for observed and effective alleles as well as for H_O, H_E and I. Based on the UPGMA algorithm, the majority of the Chinese accessions grouped in one cluster, whereas all the Korean accessions grouped together in a separate cluster, indicating that Chinese accessions are genetically quite distinct from Korean accessions. No relation between genetic relatedness among Job's tears accessions and their place of collection was observed. Chinese accessions exhibited greater within population polymorphism (P = 95 %, H_E = 0.30, I = 0.52) than the accessions from Korea (P = 68 %, H_E = 0.13, I =0.24), indicating their potentiality as a reservoir of novel alleles for crop improvement. However, in general the low diversity within each population indicates a narrow genetic base within our collection.

Additional key words: heterozygosity, Job's tears, polymorphism, simple sequence repeats.

Introduction

Coix lacryma-jobi L., commonly known as Job's tears, is a distant relative of maize in the Maydeae tribe of the grass family *Poaceae*. The annual grass is native to Burma, China, India, and Malaya, where it is grown as food source. Minimal genetic characterization has been done and little effort has been made for its improvement. Several DNA-based marker systems such as restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and micro-satellites (Mohan *et al*. 1997, Narasimhan *et al*. 2006, Dikshit *et a*l. 2007) are available now, which can accurately assess the degree of genetic diversity and relationships among accessions within a germplasm collection of any crop species. Among these, micro-satellites or simple sequence repeats (SSRs) have emerged as simple but powerful PCR-based DNA markers for genomic analysis, and in the estimation of genetic diversity held in germplasm collections of several cereal crop species including wheat (Yifru *et al*. 2006), barley (Matus and Hayes 2002), wild rice (Zhou *et al*. 2003, Gao 2005), sorghum (Dje et al. 2000), pearl millet (Budak *et al*. 2003), *etc*. There is only one report available on the use of RAPD markers to characterize genetic variation and relationships among Job's tears accessions (Li *et al*. 2001). More recently, a linkage map

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Abbreviations: AFLP - amplified fragment length polymorphism; PCR - polymerase chain reaction; RAPD - random amplification of polymorphic DNA; RFLP - restriction fragment length polymorphism; SSRs - simple sequence repeats.
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of Job's tears has been reported with the use of 80 AFLP and 10 RFLP markers in order to provide a foundation to map and isolate valuable genes (Qin *et al.* 2005). There has been no report on development and utilization of microsatellite markers for the genetic characterization of Job's tears genetic resources. Therefore, realizing the potential and need of these markers, we recently developed a set of 17 polymorphic microsatellite markers from an enriched genomic library of Job's tears (Ma *et al*. 2006). In the present study, our objective was to explore the level of genetic diversity and phylogenetic relationships within a collection of 79 accessions of Job's tears from Korea and China using 17 microsatellite markers.

Materials and methods

A total of 79 accessions of Job's tears (*Coix lacryma-jobi* L.) were collected from farms in different geographical regions of Korea and China. The collection contained 50 accessions from various regions in nine provinces of Korea and 29 accessions from the Guanxi province of China. The seedlings of each accession were grown in a greenhouse, and genomic DNA was extracted from freeze-dried fresh leaves of 15-d-old seedlings using the *Qiagen* (Hilden, Germany) DNA extraction kit. The relative purity and concentration of extracted DNA was estimated using *NanoDrop ND-1000* (Thermo Scientific, USA). The final DNA concentration was adjusted to 20 $\mu g\ cm^{-3}$.

A set of 17 primer pairs developed earlier (Ma *et al*. 2006), were used in the present study. The size of polymorphic polymerase chain reaction (PCR) products was measured accurately by following the M13 tail PCR method of Schuelke (2000). Amplification reactions were carried out in a total volume of 0.02 cm^3, containing 200 ng template DNA, 1× PCR buffer, 0.2 mM of each dNTP, 1U Taq DNA polymerase, 8 pmol of each reverse and fluorescent-labeled M13 primer and 2 pmol of the forward primer with M13 tail at its 5'end. Conditions of the PCR amplification were as follow: 94 °C (3 min), then 30 cycles each at 94 °C (30 s), 55 °C (45 s), 72 °C (1 min), followed by 10 cycles of 94 °C (30 s), 53 °C (45 s), 72 °C (1 min) and a final extension at 72 °C for 10 min. Microsatellite alleles were resolved on ABI *3130x1 Genetic Analyzer* (*Applied Biosystems*, Foster city CA, USA) using *Genescan 3.7* software and sized precisely against 6-carboxy-X-rhodamine (ROX) molecular size standards using *Genotyper 3.7* software (*Applied Biosystems*).

Basic statistics was calculated using the genetic analysis package *Popgene* version 1.31 (Yeh *et al*. 1999) for diversity measurements among 79 Job's tears accessions at each microsatellite locus, including the total number of alleles, allele frequency, accessions-specific alleles, observed heterozygosity (H_O), expected heterozygosity (H_E) and Shannon's information index (I). The same program was also used to test Hardy-Weinberg equilibrium (HWE).

The genetic similarity among accessions was determined by Dice (1945) similarity matrix. Unequivocally reproducible bands were scored and entered into a binary character matrix (1 for presence and 0 for absence). The unweighted pair group method using arithmetic averages (UPGMA) algorithm based on similarity matrix data was used to construct a dendrogram with the help of *NTSYS-pc.V.2.0* (Rohlf 1998). The genetic polymorphism of each population was assessed by calculating the mean number of alleles per locus (A), percentage of polymorphic loci (P), mean observed heterozygosity (H_O), and mean expected heterozygosity (H_E).

For the analysis of population structure and identification of ancestral and hybrid forms, we used the model-based software program *Structure* (Pritchard *et al*. 2000). In this model, a number of populations (K) are assumed to be present, each of which is characterized by a set of allele frequencies at each locus. Individuals in the sample are assigned to populations (groups), or jointly to more populations if their genotypes indicate that they are admixed. All loci are assumed to be independent, and each K population is assumed to follow Hardy-Weinberg equilibrium. The posterior probabilities were estimated using a Markov Chain Monte Carlo (MCMC) method. The MCMC chains were run at different burn-in period lengths (10 000 - 100 000) at fixed iterations of 10^6 for each fixed number of population (K). The final results were based on a burn-in period length of 70 000 and 10^6 iterations of this chain using a model allowing for admixture and correlated allele frequencies. An individual having more than 75 % of its genome fraction value was assigned to a group.

Results and discussion

Allelic diversity, frequency distributions and genetic diversity: The variability at each microsatellite locus was measured in terms of the numbers of observed and effective alleles, observed (H_O) and expected (H_E) heterozygosity and Shannon's information index (I; Table 1). All 17 loci were polymorphic, producing a total of 57 alleles among 79 Job's tear accessions; the loci varied in the numbers of observed and effective alleles from 2 to 5 and from 1.01 to 2.79 with the mean of 3.4 and 1.6 alleles per locus, respectively. The size of alleles ranged from 170 to 315 bp. The two loci GbssrJT130 and GBssr149 produced the highest number of alleles (5 in

each case) and also provided the highest and the second-highest effective number of alleles (2.79 and 2.17), as well as H_E (0.64 and 0.54) and I (1.13 and 0.92) values. The lowest number of alleles (2) was observed at each of the three loci, GbssrJT157, GbssrJT170 and GbssrJT183 with 1.02, 1.01 and 1.38 effective numbers of alleles, respectively. Significantly low values of H_E (0.01 - 0.28) and I (0.03 - 0.45) were also obtained at these three loci. In this regard, the locus GBssrJT130 was the most informative marker, whereas the locus GBJT170 was the least informative marker among 17 loci. Thus, the higher values of the effective number of alleles could be correlated with the corresponding higher H_E and I values (Table 1).

It could also be inferred from the data that microsatellites with interrupted repeats, such as

Table 1. Characteristics of 17 microsatellite loci and genetic parameters among 79 Job's tears accessions (* - locus showed significant deviation from the Hardy-Weinberg equilibrium, N_A, - number of alleles; N_E -, number of effective alleles, H_O -, observed heterozygosity, H_E - expected heterozygosity, I - Shannon's information index.

Locus name	Repeat motif	N_A	N_E	Size range [bp]	Accession-specific alleles [bp]	H_O	H_E	I
GBssrJT25*	$(GCA)_4CCA(GCA)_2$, $(TGG)_5$	3	1.757	280-296	-	0.022	0.433	0.663
GBssrJT31*	$(GCC)_5$, $(CCG)_4$	3	1.309	285-297	-	0.114	0.237	0.457
GBssrJT32*	$(TGGCTGC)_4$	4	1.964	281-295	-	0.035	0.494	0.809
GBssrJT41*	$(CTT)_3TTC(CTT)_3TTC(CTT)_2$	3	1.607	256-262	259 (C-73, China)	0.482	0.380	0.607
GBssrJT68*	$(CTCCTG)_2(CTC)_4$	4	1.695	190-271	259 (C-67, China)	0.133	0.412	0.750
GBssrJT130*	$(GGC)_6$	5	2.788	233-263	263 (C-77, China)	0.811	0.645	1.128
GBssrJT136*	$(CATG)_3$, $(CGA)_4$, $(GAG)_4$	4	1.104	223-307	-	0.024	0.095	0.246
GBssrJT149*	$(TTCAT)_4$	5	2.172	212-297	248 (K-6, Korea)	0.061	0.543	0.919
GBssrJT157	$(GCT)_4$	2	1.022	212-215	-	0.021	0.021	0.059
GBssrJT161*	$(CAT)_6$	3	1.832	206-212	-	0.189	0.458	0.707
GBssrJT164*	$(CCTCCG)_2$	3	1.213	193-221	193 (C-56, China)	0.065	0.177	0.339
GBssrJT170	$(CTG)_1T(CTG)_2CTC(CTG)_1T(CTG)_4$	2	1.011	217-223	217 (C-71, China)	0.011	0.011	0.034
GBssrJT174*	$(GAGGA)_3$	4	2.087	210-315	210 (K-26, Korea) 215 (K-37, Korea)	0.575	0.524	0.801
GBssrJT181*	$(AG)_{13}$	3	1.395	170-174		0.058	0.286	0.535
GBssrJT183*	$(CGC)_4$	2	1.385	225-228	-	0.000	0.280	0.451
GBssrJT185*	$(GGC)_5$	3	1.776	266-295	-	0.022	0.439	0.760
GBssrJT198*	$(GAT)_4GAGGA(GGAC)_3$	4	1.221	250-266	-	0.081	0.182	0.380
Mean	-	3.35	1.606	170-315	-	0.159	0.330	0.567

Table 2. Genetic parameter values at 17 microsatellite loci in the Job's tears populations from Korea and China (see Table 1 for abbreviations). Levels of polymorphism in Korean and Chinese population were 64 and 94 %, respectively.

Microsatellite locus	Korea				China			
	N_A	H_O	H_E	I	N_A	H_O	H_E	I
GBssrJT25	1	0	0	0	3	0.035	0.163	0.337
GBssrJT31	1	0	0	0	3	0.321	0.521	0.844
GBssrJT32	3	0.046	0.089	0.216	2	0.035	0.160	0.294
GBssrJT41	2	0.209	0.190	0.335	3	1	0.526	0.768
GBssrJT68	2	0.019	0.057	0.131	4	0.458	0.530	0.936
GBssrJT130	3	0.692	0.516	0.868	5	0.966	0.662	1.206
GBssrJT136	2	0.049	0.048	0.115	2	0	0.131	0.251
GBssrJT149	4	0.071	0.071	0.193	2	0	0.189	0.333
GBssrJT157	1	0	0	0	2	0.069	0.068	0.150
GBssrJT161	3	0.182	0.351	0.594	2	0.222	0.209	0.349
GBssrJT164	1	0	0	0	3	0.207	0.446	0.686
GBssrJT170	1	0	0	0	2	0.036	0.036	0.090
GBssrJT174	4	0.696	0.513	0.836	2	0.310	0.267	0.432
GBssrJT181	3	0.023	0.112	0.251	2	0.222	0.471	0.637
GBssrJT183	1	0	0	0	1	0	0	0
GBssrJT185	2	0.020	0.163	0.298	2	0	0.425	0.608
GBssrJT198	3	0.082	0.117	0.258	3	0.111	0.343	0.602
Mean	2.17	0.122	0.131	0.240	2.53	0.229	0.302	0.522

GBssrJT170 or with shorter repeat units such as GBssrJT164 and GBssrJT183 (each having four repeat unit of tri-nucleotides) were relatively less polymorphic than those with perfect repeats or with relatively longer repeat units. These results are in accordance with other reports (Depeiges *et al*. 1995, Coburn *et al*. 2002). For the majority of loci, differences in the size of alleles were observed almost exclusively by unit repeats, indicating that variations at these loci were due to differences in the number of repeat units. Three loci (GBssrJT32, GBssrJT149 and GBssrJT164) were exceptional in that allelic differences did not involve number of repeat units, indicating the possibility of different kinds of ''interruptions'' within a tandem-repeat array as well as nucleotide substitutions and insertions/deletions (indels) in regions flanking the repeat motif (Orti *et al*. 1997). 28 alleles (49.1 % of the detected alleles) were common, having frequencies between 0.05 and 0.5, 21 (36.3 %) were rare alleles, with frequencies less than 0.05 and 8 (1.4 %) were abundant alleles, with frequencies more than 0.8 (data not shown). A total of 8 unique accession-specific alleles were observed, of which five were specific to Chinese accessions and three were specific to Korean accessions. Notably, the locus GBssrJT174 provided two accession-specific alleles, of which one allele (210 bp) was specific to the accession K-26 from Jeonbuk, Korea, and the other (215 bp) was specific to the accession K-37 from Gyeonggi, Korea (Table 1). The high frequency of rare alleles (36.3 %) among Job's tears accessions (especially among Chinese accessions) indicates that they make a greater contribution to the overall genetic diversity of the collection (Roussel *et al*. 2004, Yifru *et al*. 2006). Hence, it is important to include rare alleles for maximizing the genetic variations in the gene bank collections and to utilize them in breeding (Yifru *et al*. 2006). On the other hand, unique alleles are also important because they may be diagnostic of a particular type of genotype for breeding purposes. The occurrence of a relatively higher number of unique alleles in the Chinese population indicates its potentiality as a reservoir of novel alleles for crop improvement.

Significant departure (< 0.05) from Hardy-Weinberg equilibrium was observed at all the loci with the exception of the GBssrJT157 and GBssrJT170 loci. The values of the H_O were significantly higher than the corresponding H_E at three loci; GbssrJT41, GBssrJT130 and GbssrJT174, whereas at 12 loci, the situation was the contrary (Table 1). A deficit in heterozygous individuals could be attributed to the presence of null alleles at these loci due to sequence variations at the primer sites, which prevent amplification during PCR (Lopes *et al*. 1999, Boys *et al*. 2005). The significant departure from Hardy-Weinberg equilibrium with excess homozygosity indicates a high occurrence of inbreeding. However, other factors such as mating constraints due to breeding activity and selection against heterozygotes might also be responsible for the excess of homozygous individuals.

Genetic distance-based phylogeny: Genetic similarity values were calculated from the Dice similarity index for all 79 accessions of Job's tears. The similarity coefficient values ranged from 0.11 to 1.00. These values were used to compute the UPGMA dendrogram (Fig. 1*A*). In the dendrogram 79 accessions were grouped into two main clusters at 52 % similarity. The first cluster (cluster I) contained 52 accessions with an average similarity of 89 %. It was interesting to note that with the exception of two Chinese accessions (C-61 and C-63), the cluster I included all accessions from Korea. The two Chinese accessions formed a separate sub-cluster within this cluster at 71 % similarity. It is clearly evident from the dendrogram that many accessions that were collected from the same geographical locations (*i.e*. provinces) did not cluster together, but rather, clustered with the accessions from different locations. Interestingly, some accessions from the same or from different provinces could not be distinguished from each other and seemed to be genetically identical (for example, K-1, K-2, K-17 and K-39; K-8, K-13, K-14, K-15 and K-47; K-16, K-19, K-23, K-24, K-27 and K-46, *etc*.).

The second cluster (cluster II) contained 27 accessions with an average similarity of 77 %. Notably, all accessions in this cluster were from the Guanagxi province of China. At 80 % similarity, this cluster could be further divided into four sub-clusters, of which, the first sub-cluster (IIa) contained 18 accessions, whereas 5 accessions were grouped in the second sub-cluster (IIb). Four accessions formed two sub-clusters (IIc and IId), each containing two accessions. Some Chinese accessions also could not be distinguished from each other (for example, C-52, C-54 and C-55; C-68 and C-80; C-60 and C-70), however, numbers of such genetically identical accessions were relatively lower than that of Korean accessions.

As evident from grouping of many Korean accessions from geographically different regions (provinces), no relation between genetic relatedness among Job's tears accessions and their place of collection was observed. This suggests that the origin of such accessions is not the location from which they were collected. In other words they might have originated from the same locations and seed materials might have moved from one location to another. These results clearly indicate that Chinese Job's tear's accessions were genetically distinct from that of Korean accessions.

Population genetic structure: An analysis of the genetic structure and detection of ancestral and hybrid forms among 79 individuals of Job's tears was carried out using a model-based approach (Pritchard *et al*. 2000). The inference of the exact value of K (gene pool) was not straightforward because the estimated log-likelihood values appeared to be an increasing function of K for all examined values of K. Therefore, it may not be possible to know the true value of K; in this type of situation, Pritchard and Wen (2003) suggested choosing the lowest value that captures the major structure in the data. After carefully analyzing our data for the distribution of gene

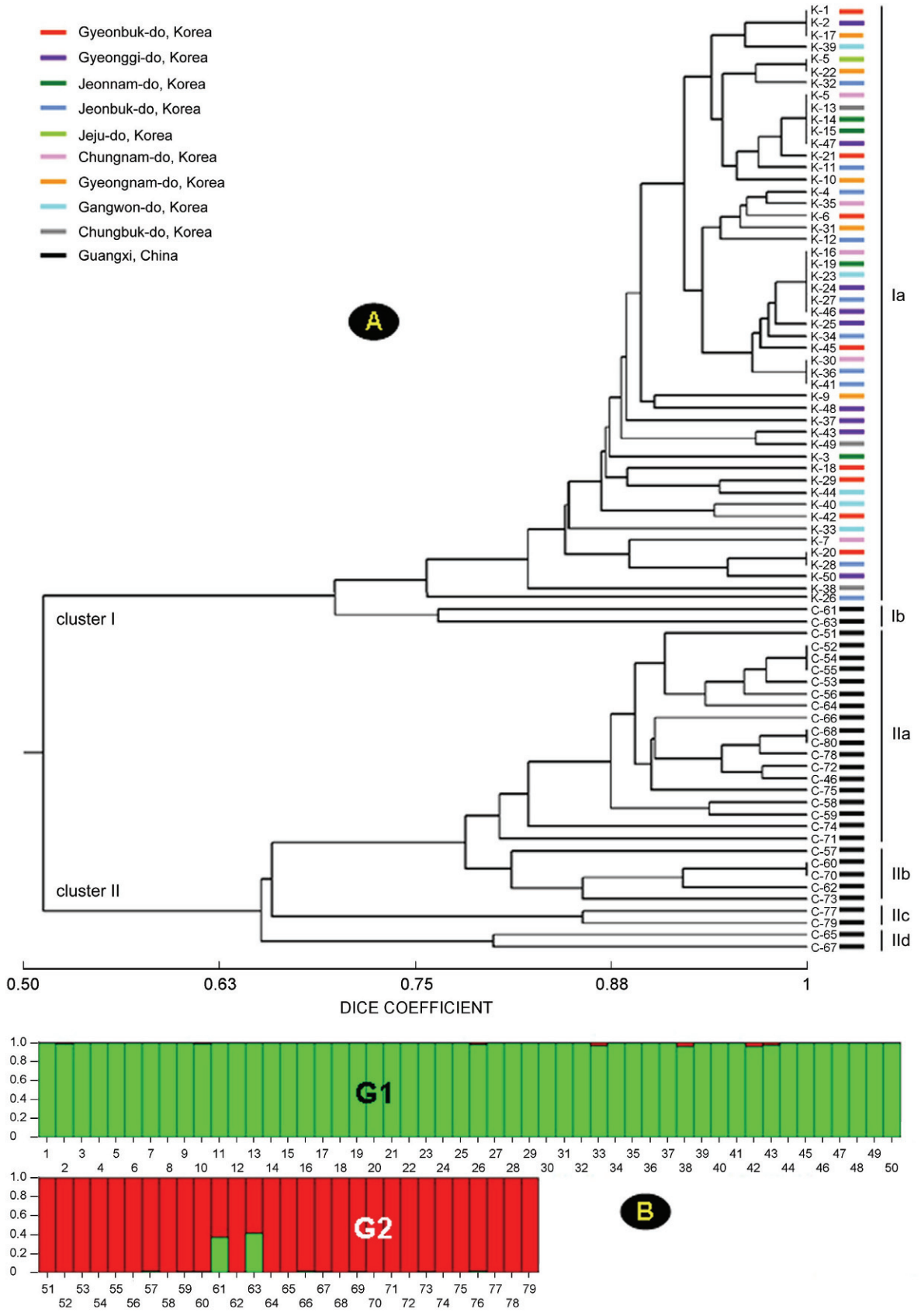
Gyeonbuk-do, Korea
Gyeonggi-do, Korea
Jeonnam-do, Korea
Jeonbuk-do, Korea
Jeju-do, Korea
Chungnam-do, Korea
Gyeongnam-do, Korea
Gangwon-do, Korea
Chungbuk-do, Korea
Guangxi, China
A
cluster I
cluster II
Ia
Ib
IIa
IIb
IIc
IId
0.50
0.63
0.75
0.88
1
DICE COEFFICIENT
G1
G2
B

Fig. 1. *A* - UPGMA dendrogram based on a Dice similarity matrix derived from 57 alleles at 17 microsatellite loci showing genetic relationships among 79 Job's tears accessions. The colored bars against each accession indicate province from where it was collected. Accessions start with K and C belong to Korea and China, respectively. *B* - Assignment of 79 Job's tears accessions to two different genetic groups (G) using *Structure* software. Each individual bar represents an accession. The green and red bars refer to two different genetic groups. The y-axis displays the estimated ancestry or membership of each accession in a particular genetic group.

pools at different values of K, we observed a biological informative pattern at K = 2 and two distinct genetic groups (G1 and G2) were identified (Fig. 1*B*). Close study of the ancestry of each individual accession revealed clear cut distribution of Korean and Chinese accessions in two separate genetic groups having ~99 % memberships of that group (Korean and Chinese accessions belong to G1 and G2, respectively). Only two Chinese accessions, C-61 and C-63 were found to be hybrid types, sharing their ~40 % and ~60 % memberships of G1 and G2, respectively. It should be noted that these two Chinese accessions were clustered with Korean accessions in the dendrogram (Fig. 1*A*). The results from two different approaches, distance-based as well as model-based analyses complement each other and provided a robust analysis.

At population level, the population from China exhibited significantly greater level of polymorphism (P = 94 %) than the population from Korea (P = 64 %; Table 2). Besides, the mean values of observed alleles, H_O, H_E and I were also significantly higher (N_A = 2.5, H_O = 2.3, H_E = 0.3 and I = 0.522) than those of Korean population (N_A = 2.2, H_O = 0.12, H_E = 0.13 and I = 0.24; Table 2), This indicates that accessions originating from China possessed greater genetic diversity than the accessions originating from Korea. Though the origins of these two genetically distinct groups are not clear, the results indicate that Chinese and Korean Job's tears accessions might be originated from two distinct gene pools. According to Arber (1965), Job's tears was introduced to China in the first century A.D. by a Chinese general who carried the seeds back to China after conquering Tongking (in Vietnam), where the grains were widely used as a cereal. Though the Chinese accessions are genetically distinct from Korean accessions and exhibited significantly higher diversity values, the overall high value of the similarity coefficient and lower values of diversity statistics within each population indicate the existence of a narrow genetic base of Job's tears accessions from two countries. Based on these observations, we suggest a wider survey and collection of Job's tears accessions from different eco-geographical regions of the world in order to conserve its maximum diversity. In addition, due to low ability of these markers in distinguishing many accessions from one another, it would also be desirable to increase the number of microsatellite markers used and or to utilize other DNA-based markers such as AFLP.

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Modern plant breeding uses many scientific and technological disciplines. Often it is difficult to know the precise meaning of many terms and to interpret accurately specific concepts. Plant breeding is a rapidly developing subject, particularly due to the new achievements in modern genetics and genetic engineering. Therefore, the second revised edition of the dictionary is very desirable.

This book provides a representative selection of more than 9400 technical terms (over 35 % more than the first edition) from the huge vocabulary of plant breeders, seed producers as well as researchers in related fields. Different terms are arranged alphabetically on a word-by-word basis. Explanation to a given term are more or less extensive, but always reflected latest discoveries in cytogenetics, molecular genetics, marker-assisted selection and experimental gene transfer. Cross-references are included when necessary. Very useful part is separate dictionary of important crop plants, weeds, ornamentals, trees, *etc*., showing their common names, description, chromosome number, genome constitution, and other details. The text is accompanied by 52 tables and 59 figures, which help reader to better orientation in this challenging discipline.

I am convinced that this dictionary will be certainly included in many reference libraries all over the world.

J. POSPÍŠILOVÁ (*Praha*)