Molecular Distinction Among Mulberry (*Morus* Spp.) Species and Varieties Cultivated in DPR Korea

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Abstract

Mulberry (*Morus* spp.) is a cross-pollinating and highly hybridized plant of which productivity are greatly varied in different varieties. We analysed molecular distinction among four mulberry species and varieties cultivated in DPR Korea by using nuclear ribosomal internal transcribed spacer (*ITS*) sequences and inter simple sequence repeat (ISSR) markers. *ITS* sequences didn't represent a remarkable interspecific distinction among four mulberry species used in our study, suggesting that it could not be employed to identify them. ISSR analysis using 16 random primers generated 158 different markers ranging from 100 to 4000 bp in size. The results showed the inter-specific genetic variation (55.34%) was slightly higher than intra-specific genetic variation (44.66%), with comparatively low average number of migrants per generation (N_m) among populations (0.3886). Using ISSR primers selected in this study, in the future, the suitable breeding strategy might be established in raising of elite mulberry varieties on the basis of interspecific hybridization.

1. Introduction

Mulberry (*Morus* spp.) is a cross-pollinating and highly hybridized plant belonging to the genus *Morus*, which is widely distributed in nature, having been evolved by natural crossing, artificial hybridization and mutation.

Mulberry is a sole food plant, of which productivity is varied in different varieties with the intraspecific distinction value of 169.8% in the early years of the last century (1928–1930), whereas 253.5% in recent years (2013–2019). Therefore, the importance of elite mulberry varieties for high silk cocoon and mulberry leaf production is much higher than any cultivation conditions. Hence, it is substantial to reorder collectively and classify more scientifically many cultivated mulberry varieties in order to produce a large number of new varieties by using good resources efficiently and subsequently to contribute to the silk cocoon production.

The genus *Morus*, which is widely distributed in tropical, sub-tropical and temperate regions, is cultivated extensively in East, Central and South Asia such as DPR Korea, China, Vietnam and Thai for silk production. The genus *Morus* has 68 recognised species including *M. mongolica* Schneid., *M. bombycis* Koidz., *M. alba* Linn., *M. multicaulis* Perr., *M. nigra* L., *M. rubra* L., *M. tartarica* L., *M. indica* L., *M. papyrifera* and *M. tinctoria* (Sanjappa, 1989).

In DPR Korea *M. mongolica* Schneid. is cultivated in mountainous areas of South and North Phyongan provinces, north Hamgyong province, Kangwon Province, Jagang Province and it exhibits high cold tolerance, having remarkably long style (1 ~ 2mm) in female flowers.

M. bombycis Koidz. is grown naturally in the mountains and fields of DPR Korea, China and Japan, being dioecious or androgynous and having similarly long style (1 ~ 2mm) like *M. mongolica* Schneid.

M. alba Linn. is most widely distributed in the world such as DPR Korea, China (Chang, 1984), Vietnam (Chang, 1984; Kansumata, 1973), India (Dhar and Ahsan, 1989; Ravindran et al., 1997) and Japan (Maichii, 1999), and is a only cultivar in European countries. This species is dioecious or androgynous and the style in female flowers is absent or indistinct.

M. multicaulis Perr. is originated from China and has been cultivated during many years in DPR Korea. It is dioecious and shown only female plants in DPR Korea with a very short style (0.2mm).

So far, using morphological and biochemical characters (Mala et al., 1998; Fotedar and Dandin, 1998; Vijayan et al., 1999; Tikader and Roy, 2001) there were many reports to classify mulberry species and cultivars, but these methods were time consuming and even might be made a mistake because of environmental variation.

Generally, the classification of mulberry is performed by comparing some morphological traits such as floral characters. For example, the length of catkins (4.1 ~ 7.2cm in some varieties while 1.7 ~ 2.4cm in other varieties) (Das et al., 1970; Tikader et al., 1995) and the style length in female flowers and the nature of stigma in male flowers (1 ~ 2mm in *M. mongolica* Schneid. and *M. bombycis* Koidz. whereas absent or 0.2 ~ 0.5mm in *M. alba* Linn. and *M. multicaulis* Perr.) (Koidzumi, 1917). But such floral characters are greatly changed depending on environmental conditions including temperature, day length and daylight, and cultivating conditions such as hormonal application or pruning of branches of the plant (Das and Mukherjee, 1992).

As above mentioned, in mulberry floral characters could not be utilized to identify species and cultivars as an only diagnostic marker. Especially, it is difficult to identify species and cultivars in highly hybridized plants like mulberry. So it is critical to use not only morphological and biochemical traits but also molecular markers to solve disruptions in species, cultivation classification and identification.

Additionally, using molecular markers the accurate classification is important in the breeding of a cross pollinating mulberry plant. Because the cross hybridization between mulberry species represent a high seed fertility (Das and Krishnaswami, 1965; Dandin et al., 1987), the interspecific crossing is favor to mulberry breeding to increase leaf yield potential.

Earlier studies have been focused on elucidation of genetic distinction among mulberry species and varieties by molecular markers (Bhattacharya and Ranade, 2001; Vijayan, 2003; Vijayan et al., 2004^{a, b, c}; Vijayan et al., 2005; Vijayan et al., 2006; Kar et al., 2008).

There were reports on phylogenetic relationship between a little number of local indian genotypes using ISSR markers (Vijayan and Chatterjee, 2003), eleven species of *M. alba* L. by ISSR and RAPD technique (Prem et al., 2004), and Chinese wild and cultivated mulberry cultivars based on ISSR and SSR markers (Zhao et al., 2006, 2007^{a, b}). By employing RAPD markers, the work has been also performed to reveal genetic relationship among some mulberry genotypes (Bhattacharya and Ranade, 2001; Chatterjee et al., 2003; Muzaffer et al., 2012; Nataraj and Chikkaswamy 2017). And recently, there were attempts to solve the problems remaining in the classificational identification of mulberry species based on RAPD and ISSR markers (Chikkaswamy and Rabin Chandra, 2014; Girish Naik et al., 2015; Rita et al., 2016).

Besides, *ITS* sequence has been used as a potential nuclear DNA marker to discriminate each other on the level of species in most plants (Walker and David, 2012). Recent study revealed that the nuclear ribosomal *ITS* sequence was more efficient than the chloroplastic *psbA-trnH* sequence in differentiating medicinal plant *Boerhavia diffusa* from other *Boerhavia* species (Dhivya et al., 2012). So far, there have been some attempts to use *ITS* sequences for phylogenetic classification and evolutionary research (Zhao et al., 2005; Zeng et al, 2015; Yahui et al., 2019).

This study aimed to elucidate genetic distinction among four mulberry species such as *M. mongolica* Schneid., *M. bombycis* Koidz., *M. alba* Linn. and *M. multicaulis* Perr., including fourteen varieties by using *ITS* sequence and ISSR markers.

Variable sites of *ITS* sequences differing in species level among 4 mulberry species used in our study were not found and it shows that *ITS* sequences can't be used to classify these mulberry species.

ITS sequences didn't show a considerable distinction among four studied mulberry species, indicating that it could not be applied to classify mulberry species.

In *M. multicaulis* Perr. the highest *ISSR* polymorphism (31.62%) and observed number of alleles (1.34), the highest effective number of alleles (1.27), Shanon's index (0.21) and Nei's gene diversity (0.15) were founded. Nei's genetic distance is the most genetically close between *M. mongolica* Schneid. and *M. bombycis* Koidz. (0.1377), while the most distant between *M. bombycis* Koidz. and *M. multicaulis* Perr. (0.3244). *ISSR* results showed that inter-specific genetic variation (55.34%) is somewhat higher than intra-specific genetic variation(44.66%), with relatively low average number of migrants per generation (*N_m*) among populations (0.3886).

This result suggests that *ISSR* markers might be superior to *ITS* sequences to distinct different species as well as mulberry varieties used in this study.

Depending on the genetic distinction among fourteen mulberry varieties cultivated in DPR Korea obtained in this study, in the future the selection of more suitable crossing pairs will be conducted to expect a desirable hybrid vigor in the breeding of a highly heterozygous mulberry plant.

2. Materials And Methods

2.1. Plant material

We used four species, fourteen mulberry varieties cultivated in DPR Korea as the experiment samples (Table 1).

Table 1 Characteristics of mulberry varieties used in this study

No	Variety	Style length/mm	the fruit of the mulberry /mm×mm	Country of origin	Species
1	"Sunchon"	1.5~ 2.0	15×10 black	DPR Korea	<i>M. mongolica</i> Schneid.
2	"Jasan"	1.5~ 2.0	15×10 black	DPR Korea	
4	"Cha1"	0.8~1.0	10×10 red or black	DPR Korea	<i>M. bombycis</i> Koidz.
3	"Gomji"	1.0	14×10 black	DPR Korea	
5	"Huichon"	1.5~2.0	15×10 black and red	DPR Korea	
6	"Sariwon1"	1.2	15×10 black	DPR Korea	<i>M. alba</i> Linn.
7	"Jam1"	0.1	14×9 white	Russia	
8	"Dongrim10"	0.2	14~16×11~9 black	DPR Korea	
9	"Dongrim316"	0.1~0.2		Japan	
10	"Ryongchon"	0.1~0.2	12×8 black	DPR Korea	
11	"Ropong"	0.2mm	26×10 black	DPR Korea	<i>M. multicaulis</i> Perr.
12	"Horo"	Absent		China	
13	"Huangro"	Absent	10×8 black	China	
14	"Sariwon2"	Absent		DPR Korea	

2.2 Genomic DNA extraction

Genomic DNA was extracted from 100 mg of fresh leaf material modifying CTAB method (Cetyl Trimethyl Ammonium Bromide) developed by Doyle & Doyle (1990). In this method the concentration of CTAB was raised up to 3 % because of mulberry leaves contain high contents of carbohydrates and polyphenols.

2.3. Assessment of DNA quality

DNA quality was measured by using Nanodrop Spectrophotometer (Thermoscientific Nanodrop 1000, USA) and subsequently performed electrophoresis through 1% agarose gel using 1X TAE buffer at 80 V for 40 mins.

2.4. Sequencing of nuclear ribosomal *ITS* sequences

ITS1, *5.8S*, *ITS2* sequences were amplified using ITS5/ITS4 primers (F: GGAAGTAAAAGTCGTAACAAGG, R: TCCTCCGCTTATTGATATGC), with genomic DNA from fourteen mulberry varieties as a temple (White et al., 1990).

PCR products were loaded onto 1.5% agarose gel for electrophoresis followed by purification using Qiagen's PCR mini-prep kit and DNA sequencing by Sanger dideoxy method.

Using MEGA 5.05, we aligned common regions (576bp) of the sequences from 14 mulberry species and 26 sequences (*M. alba* L.: AY345157, AY345148, AY345149, FJ599759, FJ917003, FJ980402, HM747164, HQ144172, HQ144173, HQ144174, JN115037, JN407491, *M. mongolica* Schneid.: AY345158, AB604250, AB604256, AB604264, AB604266, AB604268, AB604273, *M. multicaulis* Perr.: AY345153, AM042003, *M. bombycis*.Koidz: AY345151, AB604246, AB604254, AB604270, AB604282) registered to NCBI data base and analysed for their similarity.

On the basis of above aligning results, we tested the existence of *ITS* SNP pattern shared by individuals of same species to assess whether *ITS* sequences could be used to species distinction of mulberry. In case there exist a few indels peculiar to some registered sequences (AY345157, AY345149, AY345158, AY345151, AY345153), we thought these are endemic to ecological type of the species and excluded from analysis.

2.5. ISSR analysis

According to the protocol described by Gulab et al., (2018), PCR was performed in a 20µl reaction volume containing 2µl of 10X Taq buffer, 2µl of dNTPs, 2µL of MgCl₂, 1µl of primer, 0.1µL 0.1U/µL *Taq* polymerase, 1µl of temple DNA (100 ng/ µl) and 11.9 µl of ddH₂O for each sample in a Mastercycler (nexus gradient).

After 80 from ISSR primer set (UBC = University of British Columbia) on two individuals were tested for each of the four mulberry species, and we selected 16 primers (Table 3) with both reproducible and polymorphic variation with well defined and darkly staining bands.

PCR programme in Mastercycler (nexus gradient) was as follows; an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 45 s at 50°C and 2 min at 72°C, a final extension step of 72°C for 5 min.

PCR products were separated on 1.2% agarose gels in 1×TAE buffer at 80 V for 40 mins, subsequent staining gels with ethidium bromide and being taken photographs under UV light by using Geldoc-It™ (USA).

2.6. Data analysis

To analyse the genetic distinction among mulberry varieties, Nei' s genetic distances were calculated, and a dendrogram was constructed using the unweighted paired group method of cluster analysis using arithmetic averages (UPGMA) and obtained PCA plots with NTSYSpc version 2.20 (Rohlf, 2008).

The POPGENE ver. 1.32 (Yeh et al., 2000) was used to estimate values meaning genetic diversity: number of polymorphic band, observed number of alleles, effective number of alleles (Kimura and Crow 1964), Nei's (1973) gene diversity (*h*), Shannon's information index (*I*) (Lewontin 1972) and also, *G_{ST}* representing population differentiation and gene flow (*N_m*) among populations were estimated.

Analysis of molecular variance (AMOVA) was performed to evaluate the distribution of genetic variation within and among mulberry speices using the Arlequin ver. 3.5. (Excoffier & Lischer, 2010).

3. Results And Discussion

3.1 Assessment of the potential to classify four mulberry species using ITS sequences

We sequenced the nuclear ribosomal *ITS1*, 5.8S *rRNA*, and *ITS2* sequences and examined whether they were utilized to classify four mulberry species (Table 1).

Table 2

Multiple alignment of ITS sequences from 14 mulberry cultivars and previously accessed ones. (Sites indicated in the Table are variable sites differing from common sequence, Arabical numbers from 1 to 14 represent the number of each cultivar listed in Table 1.)

Species	Accession number	18	93	114	121	169	546	562	570	571	572
<i>M. alba</i> Linn.	AY345148	A	C	C	C	T	C	T	C	T	C
	FJ599759	.	.	.	.	.	.	.	.	.	.
	FJ917003	.	.	.	.	.	.	.	T	C	T
	FJ980402	.	.	.	.	.	.	.	.	.	.
	HM747164	.	.	.	.	.	.	.	.	.	.
	HQ144172	.	.	.	.	.	.	.	.	.	.
	HQ144173	G	.	.	.	.	T	.	.	.	.
	HQ144174	.	.	.	.	.	.	.	.	.	.
	JN115037	.	.	.	.	.	.	.	.	.	.
	JN407491	.	.	.	.	.	.	.	.	.	.
	8	.	.	.	.	.	.	.	.	.	.
	9	.	.	.	.	.	.	.	.	.	.
	10	.	.	.	.	.	.	.	.	.	.
		.	.	.	.	.	.	.	.	.	.
<i>M. mongolica</i> Schneid.	AB604250	.	.	.	.	.	.	.	.	.	.
	AB604256	.	.	.	.	.	.	.	.	.	.
	AB604264	.	.	.	.	.	.	.	.	.	.
	AB604266	.	.	.	.	.	.	.	.	.	.
	AB604268	.	.	.	.	.	.	.	.	.	.
	AB604273	.	.	.	.	.	.	.	.	.	.
	1	.	.	.	.	.	.	.	.	.	.
	2	.	.	.	.	.	.	.	.	.	.
	4	.	.	.	.	.	.	G	.	.	.
		.	.	.	.	.	.	.	.	.	.
<i>M. multicaulis</i> Perr.	AM042003	.	.	.	.	.	.	.	.	.	.
	11	.	.	.	.	.	.	.	.	.	.
	12	.	.	.	.	.	.	.	.	.	.
	13	.	.	.	.	.	.	.	.	.	.
	14	.	.	.	.	.	.	.	.	.	.
<i>M. bombycis</i> Koidz.	AB604246	.	G	G	T	A	.	.	.	.	.

Species	Accession number	18	93	114	121	169	546	562	570	571	572
	AB604254	.	.	.	.	.	.	.	.	.	.
	AB604270	.	.	.	.	.	.	.	.	.	.
	AB604282	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.
	5	.	.	.	.	.	.	.	.	.	.
	6	.	G	G	T	A	.	.	.	.	.
	7	.	.	.	.	.	.	.	.	.	.

In general, *ITS* sequence has been used as a potential nuclear DNA marker for phylogenetic classification and evolutionary research (Zhao et al., 2005; Walker and David, 2012; Zeng et al, 2015; Yahui et al., 2019), because it was more efficient than the chloroplastic sequences in identifying different species (Dhivya et al., 2012).

Zhao et al., (2005) constructed the phylogenetic tree of 9 mulberry species using *ITS* sequences and reported its usefulness for species identification. Zeng et al., (2015) identified 8 mulberry species species in genus *Morus* based on *ITS* sequences. Yahui et al., (2019) reconstructed phylogenetic tree of mulberry species considering pseudogenes existing in *ITS* sequences. As shown in Table 2, species specific distinction of *ITS* sequences among 4 mulberry species, *M. alba* Linn., *M. mongolica* Schneid., *M. multicaulis* Perr., *M. bombycis*. Koidz., was not found. The SNPs peculiar to 5 sequences (FJ917003, HQ144173, No 4, AB604246, No 6) were not species specific.

This suggests that a universal marker for species identification in most plants, *ITS* sequences (Walker and David, 2012; Dhivya et al., 2012) might not applied to 4 mulberry species used in this study.

3.2. Molecular distinction among mulberry species and varieties by ISSR analysis

Here we analysed the genetic relationship among and within mulberry speies were analysed by 16 informative ISSR primers (Table 3, Fig. 1).

Table 3
Primers used for ISSR analysis, total number of scored fragments
and their size range

No	Primer	Sequence (5'→ 3')	Fragment size range (bp)	Total number of bands
1	UBC807	(AG) ₈ T	100~2000	10
2	UBC808	(AG) ₈ C	100 ~ 1500	13
3	UBC810	(GA) ₈ T	200 ~ 3000	9
4	UBC811	(GA) ₈ C	300~2000	10
5	UBC812	(GA) ₈ A	200 ~ 3000	10
6	UBC823	(TC) ₈ C	100 ~ 2000	9
7	UBC825	(AC) ₈ T	500 ~ 4000	10
8	UBC827	(AC) ₈ G	250 ~ 3000	8
9	UBC830	(TG) ₈ G	100 ~ 4000	9
10	UBC834	(AG) ₈ YT	100~2000	11
11	UBC841	(GA) ₈ YC	100~4000	10
12	UBC844	(CT) ₈ RC	250 ~ 3000	9
13	UBC845	(CT) ₈ RG	300 ~ 4000	9
14	UBC852	(TC) ₈ RA	100 ~ 3000	9
15	UBC853	(TC) ₈ RT	500~3000	10
16	UBC854	(TC) ₈ RG	200 ~ 4000	12

ISSR analysis using 16 random primers generated 158 different markers ranging from 100 to 4000 bp in size. Fifty four of these were polymorphic (94.9%) over all studied genotypes, with an average of 9.88 markers per primer.

Lines marked by numbers (1, 2, 3..., 14) represent the individuals that belong to varieties listed in Table 1. M – 1 kb ladder

The genetic distinction among 14 mulberry varieties was evaluated using NTSYSpc2.11 on the basis of ISSR profiles.

Table 4
Genetic distance between 14 studied mulberry varieties

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0													
2	0.09	0												
3	0.14	0.15	0											
4	0.14	0.16	0.12	0										
5	0.21	0.18	0.15	0.23	0									
6	0.19	0.20	0.04	0.16	0.11	0								
7	0.24	0.17	0.26	0.15	0.21	0.26	0							
8	0.23	0.20	0.25	0.21	0.27	0.25	0.11	0						
9	0.29	0.21	0.30	0.21	0.24	0.26	0.08	0.10	0					
10	0.19	0.21	0.30	0.26	0.28	0.30	0.12	0.10	0.14	0				
11	0.29	0.25	0.30	0.21	0.33	0.30	0.19	0.21	0.22	0.27	0			
12	0.29	0.25	0.30	0.18	0.37	0.30	0.12	0.21	0.18	0.18	0.07	0		
13	0.35	0.26	0.32	0.22	0.29	0.27	0.24	0.26	0.28	0.37	0.15	0.19	0	
14	0.35	0.31	0.42	0.23	0.34	0.37	0.17	0.23	0.16	0.20	0.16	0.16	0.21	0

After obtaining data matrix (Table 4) based on Nei (1973)'s genetic distance, we constructed a dendrogram showing genetic relatedness of all studied mulberry varieties using UPGMA method.

As shown in Fig. 2 and Fig. 3, at genetic distance of 0.186, the dendrogram grouped all varieties in three main clusters. First cluster comprised of No. 1, 2, 4 varieties (*M. mongolica* Schneid.), and No. 3, 6, 5 varieties (*M. bombycis* Koidz.). Second cluster contained No. 7, 9, 8, 10 varieties (*M. alba* Linn.), and final third cluster involved No. 11, 12, 14, 13 varieties (*M. multicaulis* Perr.). The genetic distance among all fourteen varieties varied from 0.04 (between "Gomji" and "Sariwon 1") to 0.37 (between "Sariwon 1" and "Sariwon 3", "Ryongchon" and "Huangro") with average value of 0.22.

Based on the genetic distances between fourteen varieties, we analysed genetic relationship among four species using PopGene.version 1.31.

Table 5
Nei's (1978) genetic distance among four studied mulberry species.

	<i>M. mongolica</i> Schneid.	<i>M. bombycis</i> Koidz.	<i>M. alba</i> Linn.	<i>M. multicaulis</i> Perr.
<i>M. mongolica</i> Schneid.	*	0.8714	0.8249	0.8144
<i>M. bombycis</i> Koidz.	0.1377	*	0.7792	0.7229
<i>M. alba</i> Linn.	0.1925	0.2494	*	0.8158
<i>M. multicaulis</i> Perr.	0.2053	0.3244	0.2035	*
Nei's genetic identity (above diagonal) and genetic distance (below diagonal).				

And we analysed genetic differentiation among four mulberry species using PopGene.version 1.31.

Table 6
Genetic diversity indices for four mulberry varieties. N_{PB} - number of polymorphic bands, P_{PB} - percentage of polymorphic bands, n_a – observed number of alleles, n_e –effective number of alleles, h - Nei's (1987) gene diversity, I - Shannon's information index

Species	N_{PB}	P_{PB}	n_a	n_e	h	I
<i>Morus mongolicac</i> .K. Schneid	34	25.00	1.25 ± 0.44	1.15 ± 0.30	0.09	0.21
<i>Morus bombycis</i> .Koidz	25	18.38	1.18 ± 0.39	1.10 ± 0.24	0.06	0.10
<i>Morus alba</i> . Linn	34	25.00	1.25 ± 0.44	1.19 ± 0.36	0.11	0.15
<i>Morus multicaulis perr</i>	43	31.62	1.34 ± 0.48	1.27 ± 0.40	0.15	0.21

We obtained a mean *Gst* value of 0.5627 for 4 mulberry species (ranging from 0.0803 to 0.8290) and an average number of migrants per generation (N_m) among populations of 0.3886 (in the range of 0.1031 and 5.7241).

Finally, we performed AMOVA to estimate genetic differentiation among 14 mulberry varieties cultivated in DPR Korea using Arlequin ver. 3.5.2.2.

Table 7
Molecular analysis of variance (AMOVA) based on ISSR profiles in four mulberry species

Source of variation	degrees of freedom	Sum of squares	Variance components	Percentage of variation (%)	P	Fixation Indices
Among species	3	204.881	3.51667 Va	55.34	$P < 0.0002$	F_{CT} 0.45345
Within species	10	175.833	2.91758 Vb	44.66	$P < 0.0002$	F_{SC} 1.0000
Total	13	385.714	6.43425			

As shown in Table 7, of the total genetic diversity, 55.34 % was attributable to differences among species and 44.66% was to differences between varieties, showing higher interspecific differentiation than varietal differentiation.

ISSR or RAPD technique has been utilized in elucidation of genetic distinction among mulberry species and varieties.

In previous studies, 8 RAPD primers produced 67 discrete bands varied from 300 to 1000 bp showing 93.1% polymorphism among 14 mulberry genotypes (Rita et al., 2016), 16 primers generated 61 polymorphic bands of 75 bands ranging from 300 to 5000 bp showing over 18 mulberry cultivars with an average of 4.6 markers per primer (Chikkaswamy and Rabin, 2014). And Shivashankar (2015) obtained 41 of 45 amplication products (94% polymorphism) with 300–5000 bp in size, confirming the tendency similar with our results.

In Prem et al. (2004)'s study on *M. alba* Linn., the genetic similarity was in the range of 0.738 for China whitw and Kattaneo to 0.909 for Sujampur-2 and Punjab local with a average of 0.834, suggesting the tendency similar to our results.

As shown Table 5 and Fig. 4, the group of *M. mongolica* Schneid. was the most genetically close to the group of *M. bombycis* Koidz. (genetic distance- 0.1377), and the latter was the most genetically distant to the group of *M. multicaulis* Perr. (genetic distance- 0.3244), reflecting somewhat their geographic origin. Our results show similar tendency with the genetic similarity coefficient among *M. alba* Linn., *M. bombycis* Koidz., and *M. multicaulis* Perr. species in Awasthi (2004)' study (*M. alba* Linn. and *M. multicaulis* Perr.: 0.573 > *M. alba* L. and *M. bombycis* Koidz. : 0.405, *M. bombycis* Koidz. and *M. multicaulis* Perr. : 0.385).

In Prem et al. (2004)'s study on white mulberry species, Indian genotypes made a distant group with the Japanese, indicating the availability of Japanese genotypes in the genetic improvement of Indian white mulberry geneotypes. Similarly, in our work *M. mongolica* Schneid. and *M. bombycis* Koidz. resulted in a distant group with *M. alba* L. and *M. multicaulis* Perr., suggesting the possibility of cross hybridization between these species in the breeding of elite mulberry varieties.

In this study, the highest ISSR polymorphism (31.62%) and observed number of alleles (1.34), the highest effective number of alleles (1.27), Shanon's index (0.21) and Nei's gene diversity (0.15) were obtained for *M. multicaulis* Perr. population (Table 6). Based on Nei's genetic distances, the most genetically close pair is *M. mongolica* Schneid. and *M. bombycis* Koidz. (0.1377), whereas the most distant one is - *M. bombycis* Koidz. and *M. multicaulis* Perr. (0.3244). The results showed the low intra-specific genetic variation (44.34%) and the slight high inter-specific genetic variation (55.66%).

In Zhenjiang et al., (2016)' study evaluating genetic relationship among 42 mulberry genotypes from seven countries over Asia and South America using 17 inter simple sequence repeat (ISSR) primers, the average Nei's index and Shanon's information index were 0.116 and 0.174, respectively, showing low diversity of mulberry. This exhibits a similar tendency with the results on *M. alba* L. (Nei's index-0.11, Shanon's information index-0.15) in our study.

Conclusions

M. mongolica Schneid., *M. bombycis* Koidz., *M. alba* L. and *M. multicaulis* Perr. used in this study are cultivated extensively for sericulture in DPR Korea, are differentiated from each other based on the style length in female flowers. But this character can be changed according to environmental and cultivational conditions, the identification of mulberry species and varieties depending on only this character likely cause a mistake in

establishing breeding strategy of a cross pollinating mulberry plant. But *ITS* sequence commonly used for species identification in most plants can not be applied to mulberry species owing to the deficiency of interspecific distinction.

Therefore, the genetic relationship among fourteen mulberry varieties cultivated in DPR Korea obtained in this study, will be contributed to the selection of more suitable crossing pairs in the breeding of a highly heterozygous mulberry plant expecting a desirable hybrid vigor.

Declarations

The authors have no relevant financial or non-financial interests to disclose.

The authors have no conflicts of interest to declare that are relevant to the content of this article.

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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Figures

Figure 1

ISSR agarose gel electrophoresis profiles of fourteen mulberry varieties using UBC808 (left), UBC823 (middle) and UBC834 (right) primers.

Figure 2

UPGMA dendrogram illustrating the genetic relatedness between fourteen mulberry varieties based on Nei's genetic distance

Figure 3

2-D(left) and 3-D(right) PCA plot based on ISSR data in mulberry varieties.

Figure 4

UPGMA dendrogram illustrating the genetic relationships between four mulberry varieties based on Nei's genetic distance