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Analysis of Phylogenetic Relationship of 30 Cultivars of Korean Mulberry (Rosales: Moraceae) in Korea

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Abstract

This study was carried out to understand phylogenetic relationships of the 30 mulberry cultivars converved in Korea based on the ITS rDNA region, and they were compared to 40 reference sequences from GenBank. The size and the G+C content of the ITS rDNA gene regions from the 30 Korean mulberry cultivars and 40 reference sequences varied from 612–630 bp and 58.19–61.62%, respectively. Based on the results of the comparative phylogenetic analysis of the ITS rDNA regions of the 30 Korean mulberry cultivars and 40 reference sequences, they were divided into three groups (Group 1, 2, and 3) and two subgroups (Group 1A and 1B within Group 1). The sequence lengths of the Korean mulberry cultivar numbers 1–26 and 27–30 were 615 bp and 616 bp, respectively. At 205 bp location of ITS1 rDNA region, the cultivar numbers 1–26 contain the nucleotide thymine but the cultivar numbers 27–30 contain the nucleotide adenine. In addition, the insertion of the nucleotide adenine at 206 bp location was found only in the four Korean mulberry cultivars (numbers 27–30). Based on these sequence information and phylogenetic result, the 30 Korean mulberry cultivars were identified as *M. alba* and *M. australis*. This study will contribute to the construction of genetic database constructions and accurate variety identifications for unidentified mulberry varieties in Korea.

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Introduction

Mulberry (*Morus* spp.) belongs to the order Rosales, family Moraceae, and genus *Morus* (Zhang *et al.*, 2011). The genus *Morus* is distributed in a wide area of worldwide including Asia, Europe, North and South America, and Africa (Awasthi *et al*., 2004). The mulberry family Moraceae, which comprises 37 genera with over 1,100 species (He *et al*., 2013), has long been used as a traditional medicine and food in Asian countries (Korea, China, and Japan) (Jeong *et al*., 2014). The mulberries are economically important to industry of silk and functional food (Zhu *et al*., 2011; Nepal and Ferguson, 2012; Jeong *et al*., 2014).

In 1753, the genus *Morus* was initially divided into seven species (*Morus alba* L., *Morus nigra* L., *Morus rubra* L., *Morus tartarica* L., *Morus indica* L., *Morus papyrifera*, and *Morus tinctoria*) by Linneaus. Among them, *M. papyrifera* and *M. tinctoria* were later shifted to *Broussonetia papyrifera* (L.) L'Hér. ex Vent. and *Maclura tinctoria*. (L.) D.Don ex Steud., respectively. Bureau (1873) described 21 varieties

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and 13 sub-varieties based on characteristics of leaves and pistillate catkins. In 1874, Brandis reported four species, and classified the genus into two sections based on style length. It also separated both the sections again based on the length and shape of the syncarp and a few leaf characters. Schneider (1917) described a new Chinese species named for *Morus notabilis* C. K. Schneid. In addition, Koidzumi (1917) reported for 24 species and one subspecies in the genus *Morus* according to the style length and the nature of the stigma for female and male flowers. After that, Leroy (1949) and Hotta (1954) divided the genus *Morus* into 19 species and 35 species, respectively. So far, the mulberries have been cited more than 150 species in the Index Kewensis. However, most of the cases of them have been treated either as synonyms or as varieties rather than species, and some have been transferred to allied genera (Sharma *et al*., 2000; Awasthi *et al*., 2004). Currently, the genus *Morus* are generally cited and accepted for only 10–16 species including *M. alba*, M. *australis*, *M. cathayana*, *M. macroura*, *M. mongolica*, *M. nigra*, *M. notabilis*, *M. serrata*, *M. celtidifolia*, *M. insignis*, *M. microphylla*, *M. rubra*, *M. mesozygia*, *M. bombycis*, *M. wittiorum*, and *M. trilobata* (Pawlowska *et al*., 2008; Kapche *et al*., 2009; Nepal and Ferguson, 2012).

Recently, several studies have been reported for the genetic diversity of *Morus* using genetic approaches. Internal transcribed spacer (ITS) has been widely used in phylogenetic study of plants because of has the highest discriminatory power for species (Àlvarez and Wendel, 2003; Li *et al*., 2011). Zhao *et al*. (2005) have investigated some Asian taxa (including *M. alba*, *M. australis*, *M. macroura*, and *M. mongolica*) according to genetic distances of the ITS region and the chloroplast *trn*L intron. In addition, Nepal and Ferguson (2012) reported phylogenetic relationships for 13 species within *Morus* genus using sequence data from ITS of the nrDNA and the chloroplast *trn*L-*trn*F intergenic spacer. More recently, Zeng *et al*. (2015) classified the *Morus* genus into eight species (including *M. alba*, *M. nigra*, *M. notabilis*, *M. serrata*, *M. celtidifolia*, *M. insignis*, *M. rubra*, and *M. mesozygia*) using DNA sequences of ITS rDNA region.

In the present study, we investigated the phylogenetic relationship from 30 cultivars of Korean mulberry by analyzing the ITS rDNA region, and were compared to the reference sequences of NCBI databases.

Materials and Methods

Sample preparation

Mulberry leaves of 30 cultivars were collected from the Sericultural and Apicultural Materials Division of the Department of Agricultural Biology, Rural Development Administration, Jeon-Ju, Republic of Korea. Fresh mulberry leaf samples were dried in an oven at 40°C for 24 hours and then were ground to a fine powder using liquid nitrogen.

DNA extraction

DNA extraction of mulberry leaf samples was using the cetyl trimethyl ammonium bromide (CTAB) method (Cao *et al*., 1998). The extracted DNA was purified with a Phenol: Chloroform: Isoamyl alcohol (25:24:1) and then precipitated with one volume of isopropanol and 1/30 volume of 3 M sodium acetate. The precipitated DNA was washed sequentially with 70% ethanol and dried. The DNA pellet was dissolved in 60 µL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The dissolved DNA was treated with 6 µL of RNase A (20 mg/mL) and incubated at 37°C for 30 mins. The extracted DNA was used as a template (adjusted to 100 ng/µL) for PCR amplification.

PCR amplification

The target sequence regions (the partial 18S rRNA gene, ITS 1, 5.8S rRNA gene, ITS 2, and the partial 28S rRNA gene) for PCR amplification were performed using forward (5'–GTAACAAGGTTTCCGTAGGTG–3') and reverse (5'–TAAACTCAGCGGGTAGCC–3') primers designed by Zeng *et al*. (2015). PCR reactions were performed with a premixed polymerase kit (Taq PreMix; TNT Research, Seoul, Korea) in a 20 µL reaction mixture containing 1 µL of DNA. The PCR amplification conditions were carried out at 5 min initial denaturation at 94°C, followed by 30 cycles of 30 sec denaturation at 94°C, 30 sec primer annealing at 56°C, and 1 min extension at 72°C in a thermal cycler (TaKaRa, Otsu, Japan). A final extension step was carried out for 10 min at 72°C. Sequences of purified PCR products were analyzed by an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) at Genocell Total Biotechnology (Yongin, Korea).

Sequence analysis

For phylogenetic analysis of 30 cultivars of Korean mulberry, the resultant sequences were compared to 40 reference sequences in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov). The sequences for phylogenetic analysis were aligned using the BioEdit program (http://www. mbio.ncsu.edu/bioedit/bioedit.html). The phylogenetic tree was constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987) that is implemented in the MEGA7 program (Kumar *et al*., 2016). The confidence levels for the individual branches of the resulting tree were assessed by the bootstrap test (Felsenstein, 1985), in which 1,000 replicate trees were generated from resampled data. *Artocarpus heterophyllus* (FJ917039) and *Broussonetia papyrifera* (HM623778) were used as outgroup in the phylogenetic analysis.

Results and Discussion

Sequence analysis of Korean mulberry based on ITS rDNA region

The sequence information for the ITS rDNA region of the Korean mulberry cultivars and reference sequences can be seen in Table 1 and Table 2, respectively. In Korean mulberry cultivars (Table 1), the sequence length and the total G+C content for the ITS rDNA (included ITS 1, 5.8S rRNA, and ITS 2) region were divided largely into two groups (cultivar numbers 1–26 and 27–30). Each the cultivar numbers 1–26 and 27–30 of the Korean mulberry had different sequence lengths in the ITS rDNA region (615 bp and 616 bp, respectively). In addition, their G+C contents showed 59.02% and 58.93%, respectively. The cultivar numbers 1–26 of Korean mulberry has the sequence size of 215 bp in ITS1, 163 bp in 5.8S rRNA, and 237 bp in ITS 2, respectively. The cultivar numbers 27–30 of Korean mulberry showed the same sequence lengths from the 5.8S rRNA and ITS 2 with cultivar numbers 1–26, whereas their ITS 1 region had the different sequence length (216 bp), which is one bp shorter length compared to the cultivar numbers 1–26.

In the 40 reference sequences of mulberry (Table 2), the total G+C contents of the ITS rDNA region were 58.19%–61.62%. The sequence lengths of the ITS rDNA region including ITS 1, 5.8S rRNA, and ITS 2 ranged from 612 to 630 bp. Among them, the shortest and longest length of the ITS rDNA sequence were *M. alba* (FJ917003) and *M. celtidifolia* (HM747168), respectively. In addition, the sizes of ITS1 and ITS 2 region varied from 212–231 bp and 221–240 bp, respectively. However, the 5.8S rRNA regions showed the same sequence length (163 bp) to *Morus* species, showing a high conservancy in length.

In previous studies, the lengths of the 5.8S rRNA region in *Morus* species were 100, 152, and 159 bp, respectively (Zhao *et al*., 2004; Nepal, 2008; Chen *et al*., 2010). Recently, Zeng *et al.* (2015) reported that the sizes of the 5.8S rRNA regions of *Morus* species including *M. alba*, *M. alba* var. atropurpurea, *M. australis*, M. *bombycis*, *M. cathayana*, *M. indica*, *M. macroura*, *M. mongolica*, *M. alba* var. multicaulis (*M. lhou*), and *M. wittiorum* were all identically 163 bp. Likewise, the 30 Korean mulberry cultivars and 40 reference sequences in our study also were confirmed to be identical in length (163 bp) in 5.8S rRNA region. Zeng *et al.* (2015) also showed that the *Morus* species, which has the 215 bp in ITS 1 sequence have identical sequences in 5.8S including *M. alba*, *M. mongolica*, *M. cathayana*, *M. australis*, *M. macroura*, *M. wittiorum*, *M. bombycis*, *M. indica*, *M. trilobata*, and *M. alba* var. multicaulis. However, in the results of our study, among the *Morus* species with a 215 bp in ITS1 sequence, the 5.8S rRNA sequences of *M. australis* (AM042004) and *M. alba* var. multicaulis (KF784883) were confirmed to have some difference.

The ITS 1 region of the Korean mulberry cultivars was found to have a substitution (A/T at 205 bp location) and deletion/insertion (–/A at 206 bp location) of a single nucleotide between the cultivar numbers 1–26 and 27–30 (Table 3). Especially, a single nucleotide substitution (A/T) at 205 bp location and insertion of the nucleotide [A] at 206 bp location were found only in the four Korean mulberry cultivars (numbers 27–30), one *M. australis* (HM747166), and one *M. notabilis* (HM747175). However, *M. notabilis* (HM747175) in ITS 2 region has difference at 597 bp location (transversion, G/T) from the four Korean mulberry cultivars (numbers 27–30) and *M. australis* (HM747166).

Phylogenetic analysis of Korean mulberry

In the phylogenetic analysis, the 30 Korean mulberry cultivars and 40 reference sequences were divided into three groups (Group 1, 2, and 3) and two subgroups (Group 1A and 1B within Group 1) (Fig. 1). Each group was supported by bootstrap analysis at 42% to 99%, respectively. All of the Korean mulberry cultivars

Table 1. Sequence information for ITS region of Korean mulberry cultivars used in this study

No.	cultivars	ITS rDNA				G+C content (%)	Species identification
		ITS 1	5.8S	ITS 2	Length (bp)		
1	Ajuguk 45	215	163	237	615	59.02	
2	Amsubomburi	215	163	237	615	59.02	
3	Baek Gwang 6	215	163	237	615	59.02	
4	Baek Hak	215	163	237	615	59.02	
5	Buk Nong 10	215	163	237	615	59.02	
6	Bu Ru	215	163	237	615	59.02	
7	Chung Mok Si Pyung	215	163	237	615	59.02	
8	Chung Sip Ryang	215	163	237	615	59.02	
9	Da Ho Jo Saeng	215	163	237	615	59.02	
10	Da Ho Chuk	215	163	237	615	59.02	
11	Il Pum Mok	215	163	237	615	59.02	
12	Jang Jam B	215	163	237	615	59.02	
13	Jang Roe	215	163	237	615	59.02	
14	Jeok Da Ho	215	163	237	615	59.02	*Morus alba*
15	Jeok Gab Chan	215	163	237	615	59.02	
16	Man Saeng Baek Pi Sang	215	163	237	615	59.02	
17	MB 1	215	163	237	615	59.02	
18	MB 2	215	163	237	615	59.02	
19	MB 3	215	163	237	615	59.02	
20	MB 4	215	163	237	615	59.02	
21	MS 1	215	163	237	615	59.02	
22	MS 2	215	163	237	615	59.02	
23	Pum Bo 2	215	163	237	615	59.02	
24	Ri Ma	215	163	237	615	59.02	
25	Sa Bang	215	163	237	615	59.02	
26	Sam Duk	215	163	237	615	59.02	
27	Dae Ya Ok Sung	216	163	237	616	58.93	
28	Dae Yup Seo Ban	216	163	237	616	58.93	
29	Gu Moon Yong	216	163	237	616	58.93	*Morus australis*
30	Jeok Mok Si Pyung	216	163	237	616	58.93	

Table 2. Information for ITS rDNA region of reference sequence (NCBI databases)

Species	GenBank Accession NO.	ITS rDNA				G+C content (%)
		ITS 1	5.8S	ITS 2	Length (bp)	
Morus alba	FJ980402, HQ144172, JN4070491, KF784881, KF784885, KF784891, KF784896, KF784897	215	163	237	615	59.02
	KF784884*	215	163	237	615	58.86
	KF784887*	215	163	237	615	58.86
	FJ917003	212	163	237	612	58.82
Morus alba var. atropurpurea	AY345145	215	163	235	613	58.89
	KF784888, KF784892	215	163	237	615	58.82
Morus alba var. multicaulis	KF784883	215	163	237	615	58.86
Morus australis	AM042004	215	163	237	615	58.86
	KF784889	215	163	237	615	59.02
	KT002555	215	163	237	615	59.35
	HM747166	216	163	237	616	58.93
Morus bombycis	AM042006	215	163	237	615	59.02
Morus cathayana	AM042001	215	163	237	615	59.02
	HM747167	215	163	237	615	59.35
Morus celtidifolia	HM747168	228	163	239	630	58.57
Morus indica	AM041997, KF784890	215	163	237	615	59.02
Morus insignis	HM747169	229	163	236	628	61.62
Morus lhou	AM041999	215	163	237	615	59.02
Morus macroura	HM747170	215	163	237	615	59.35
Morus mesozygia	HM747171	231	163	221	615	59.51
Morus mongolica	HM747173*	215	163	237	615	59.02
	KF784879*	215	163	237	615	59.02
Morus murrayana	FJ605515	229	163	237	629	58.35
Morus nigra	KF784875	228	163	237	628	58.28
Morus notabilis	HM747175	216	163	237	616	59.09
Morus rubra	HQ144180	229	163	237	629	58.19
	FJ605516	215	163	237	615	59.02
Morus wittiorum	AY345154	215	163	240	618	58.74
	KF784886	215	163	237	615	59.35

*, Although they had the same sequence length and G+C content, they differed in two nucleotide sequences.

Table 3. Sequence difference of ITS 1 region between the two Korean mulberry cultivar groups

Species	Sequence (5'–3')
FJ980402 *Morus alba*	CAGCTGTGTTGTGCTT–GGTTAAGTCTAAAA
Korean mulberry cultivars No. 1-26	CAGCTGTGTTGTGCTT–GGTTAAGTCTAAAA
HM747166 *Morus australis*	CAGCTGTGTTGTGCTAAGGTTAAGTCTAAAA
Korean mulberry cultivars No.27-30	CAGCTGTGTTGTGCTAAGGTTAAGTCTAAAA

Red highlight indicates a single nucleotide substitution between two Korean mulberry cultivars.
Blue highlight indicates insertion/deletion of a single nucleotide between two Korean mulberry cultivars.

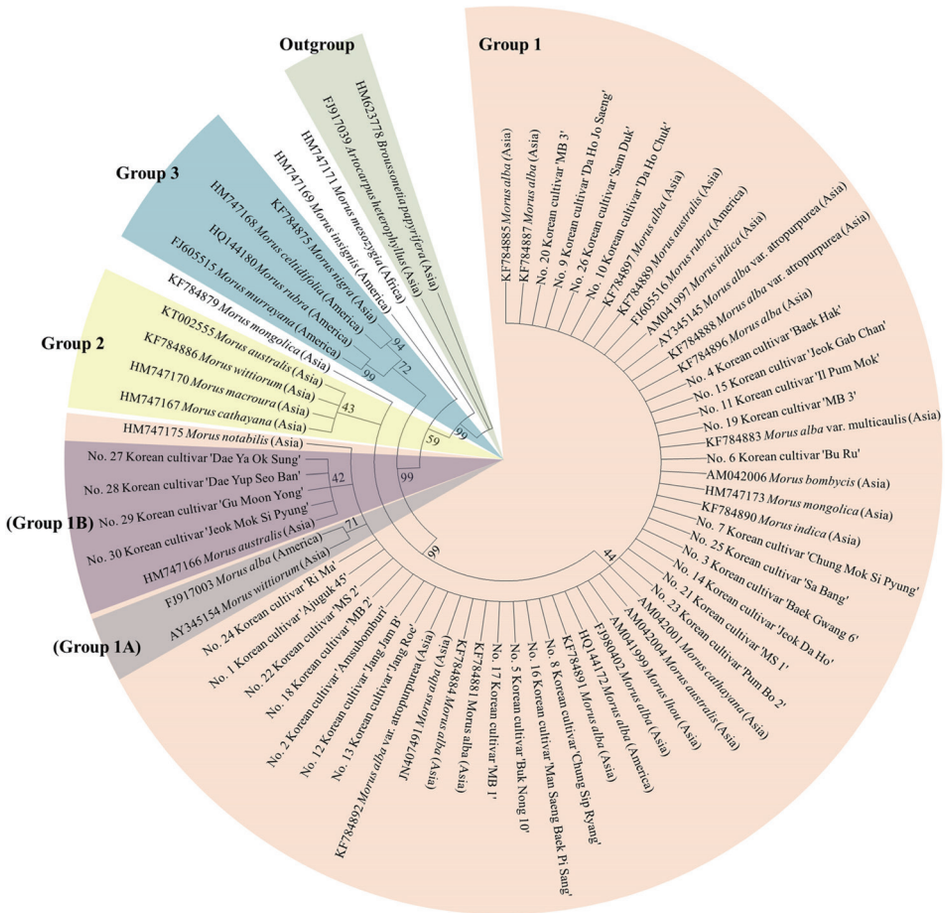

Fig. 1. Phylogenetic relationships based on ITS rDNA region sequences among 30 Korean mulberry cultivars and 40 reference sequences. The tree was obtained using the neighbor–joining method. Numbers at the branch nodes represent bootstrap values obtained from 1,000 replications. Two strains of *Artocarpus heterophyllus* and *Broussonetia papyrifera* were used as the outgroups.

clustered into Group A together with 11 *Morus alba* (FJ917003, FJ980402, HQ144172, JN407491, KF784881, KF784884, KF784885, KF784887, KF784891, KF784896, and KF784897), three *M. alba* var. atropurpurea (AY345145, KF784888, and KF784892), one *M. alba* var. multicaulis (KF784883), three *M. australis* (AM042004, HM747166, and KF784889), one *M. bombycis* (AM042006), one *M. cathayana* (AM042001), two *M. indica* (AM041997 and KF784890), one *M. lhou* (AM041999), one *M. mongolica* (HM747173), one *M. notabilis* (HM747175), one *M. rubra* (FJ605516), and one *M. wittiorum* (AY345154). This group showed 98.2%–100% identity. Group 2 included four Asian *Morus* species (*M. australis*, *M. cathayana*, *M. macroura*, and *M wittiorum*) with 99.9%–100% identity in the group and it was closely related to *M. mongolica* (KF784879). Asian *Morus*

nigra (KF784875) and three American *Morus* species (*M. celtidifolia*, *M. murrayana*, and *M. rubra*) clustered into Group 3 and this group had 97.3%–99.9% identity. American *M. insignis* and African *M. mesozygia* was closely related to Group 3.

In addition, Group 1 was divided into two subgroups (Group 1A and Group 1B). Group 1A included American *M. alba* (FJ917003) and Asian *M. wittiorum* (AY345154) with 98.4% identity. Group 1B included four Korean mulberry cultivars (No.27 'Dae Ya Ok Sung', No. 28 'Dae Yup Seo Ban', No. 29 'Gu Moon Yong', and No. 30 'Jeok Mok Si Pyung') and Asian *M. australis* (HM747166) with 100% identity.

In Group 1, the cultivars numbers 1–26 of Korean mulberry showed 100% identity with eight *M. alba* (FJ980402, HQ144172, JN4070491, KF784881, KF784885, KF784891, KF784896, and KF784897), one *M. australis* (KF784889), one *M. bombycis* (AM042006), one *M. cathayana* (AM042001), two *M. indica* (AM041997, KF784890), one *M. lhou* (AM041999), and one *M. mongolica* (HM747173), except for three *M. alba* (KF784884, KF784887, and FJ917003), three *M. australis* (AM042004, HM747166, and KF784889), two *M. australis* (AM042004 and HM747166), one *M. notabilis* (HM747175), and one *M. wittiorum* (AY345154). These phenomena could be caused because the *Morus* species of the same species, subspecies, or varieties/cultivars have been misidentified and misnamed. We found that the *Morus* species including *M. australis* (KF784889), *M. bombycis* (AM042006), *M. cathayana* (AM042001), *M. indica* (AM041997 and KF784890), *M. lhou* (AM041999), and *M. mongolica* (HM747173) could be synonym with *M. alba*. Furthermore, three *M. alba* (KF784884, KF784887, and FJ917003) could be subspecies or varieties of *M. alba*.

Morus australis is called Korean mulberry or Chinese mulberry, and commonly distributed in East Asia (included China, Japan, and Korea) and South-East Asia (Zheng *et al*., 2012). *Morus australis* has already been reported for general morphology, voucher information, collection location, specimens examination and sequences of ITS and trnL-trnF (GenBank accession numbers HM747166 and HM747182) (Nepal, 2008; Nepal and Ferguson, 2012). Vijayan *et al*. (2006) reported that *M. alba* and *M. australis* based on inter simple sequence repeat (ISSR) were closely clustered but *M. macroura* was genetically distinct. In addition, Nepal (2008) reported that *M. australis* in phylogenetic analysis based on ITS rDNA region was closely related to *M. notabilis*. In our results, *M. australis* (HM747166) within Group 1B was closely related to *M. alba* and *M. notabilis*. However, *M. australis* (KT002555) within Group 2 was closely related to *M. cathayana*, *M. macroura*, and *M wittiorum*. Thus, among four *M. australis*, those with the GenBank accession numbers AM042004, KF784889 and KT002555 had been misidentified as *M. australis*, except for that with the GenBank accession numbers HM747166. Consequently, the Korean cultivar numbers 1–26 and 27–30 of the genus *Morus* in our study could be identified as *M. alba* and *M. australis*, respectively.

The genus *Mours* was initially classified on the basis of morphological characteristics. However, the classifications based on morphological traits of *Morus* can confuse the diversity and relationships among different mulberry species because of wide geographical distribution, morphological plasticity, hybridization, and environmental influence (Awasthi *et al*., 2004; Nepal and Ferguson, 2012). The genus *Morus* can be classified into 13 species based on synthesis of taxonomic literature and examination for over 1500 herbarium specimens (Nepal, 2008); eight Asian species (*M. alba*, *M. australis*, *M. cathayana*, *M. macroura*, *M. mongolica*, *M. nigra*, *M. notabilis*, and *M. serrata*), four New World species (*M. celtidifolia*, *M. insignis*, *M. microphylla*, and *M. rubra*), and one African species (*M. mesozygia*).

In Korea, the common mulberry species are *M. alba* L., *M. indica* L., *M. bombycis* Koidz, *M. tiliaefolia* Makino, and *M. nigra* L. (Kalpana *et al*., 2012; Rao *et al*., 2013). In the present study, we analyzed the ITS rDNA region of the 30 Korean mulberry cultivars, and they were identified as *M. alba* and *M. australis*. Currently, more than six hundred mulberry varieties are found in Korea, but the majority of the varieties have still been unidentified for their taxonomic position and species name. Therefore, additional study along with the present study for unidentified mulberry varieties in Korea are needed for the construction of genetic database and accurate variety identifications for the protection of breed and subsequent breeding.

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