IJIE Int. J. Indust. Entomol. 45(1) 29-34 (2022)
ISSN 1598-3579, http://dx.doi.org/10.7852/ijie.2022.45.1.29

ISSN 1598-3579 (Print)
ISSN 2586-4785 (Online)

Identification of domesticated silkworm varieties using single nucleotide polymorphisms detected from mitochondrial genomes

Jong Woo Park[1,†], Jeong Sun Park[2,†], Chan Young Jeong[1], Sang Kuk Kang[1], Seong-Wan Kim[1], Nam-Suk Kim[1], Kee Young Kim[1], and Iksoo Kim[2,*]

[1]*Department of Agricultural Biology, National Academy of Agricultural Science, Rural Development Administration, Wanju Gun, Jeollabuk-do, 55365, Republic of Korea*
[2]*Department of Applied Biology, College of Agriculture & Life Sciences, Chonnam National University, Gwangju, 61186, Republic of Korea*

Abstract

Silkworms have recently attracted attention as healthy functional foods. Different varieties of silkworms have functional differences; thus, there is an emerging need for variety identification. In this study, we sequenced complete mitochondrial genomes (mitogenomes) of ten government-recommended silkworm varieties (BaekHwang, BaekOk, DaeBaek, DaeBak, DaeHwang, GoldenSilk, HanSaeng, JooHwang, KumKang, and KumOk). Comparison of these sequences allowed us to select the single nucleotide polymorphisms (SNPs) in 34 sites that are specific to six silkworm varieties: 13 in DaeBak, 8 in GoldenSilk, 9 in KumKang, 2 in BaekHwang, 1 in BaekOk, and 1 in DaeHwang. Among these each one SNP per variety was amplified by preparing variety-specific primers and then using tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR). As a result, it was possible to identify these six varieties among the ten silkworm varieties, evidencing that SNPs developed from mitogenomes are useful marker for the discrimination of genetically closer silkworm varieties.

Int. J. Indust. Entomol. 45(1), 29-34 (2022)

Received : 22 Aug 2022
Revised : 22 Sep 2022
Accepted : 26 Sep 2022

Keywords:
Silkworm varieties,
Molecular markers,
Single Nucleotide Polymorphism,
T-ARMS-PCR,
Mitochondrial genome

Introduction

In addition to its importance for raw silk production, the domesticated silkworm, *Bombyx mori* Linnaeus, 1758 (Lepidoptera: Bombycidae), has long been used as a nutritional and pharmacological resource (Ryu *et al.*, 2002, 2003). Currently, the silkworms that are recommended to farms by the Korean government are KumOk, DaeBak, BaekOk, DaeBaek, and KumKang, which make white cocoons; DaeHwang, BaekHwang, and GoldenSilk, which make yellow cocoons; Yeonokjam which builds green cocoons; and JooHwang, which builds orange cocoons (Lee *et al.*, 1985; Kang *et al.*, 2003, 2007, 2010; Kim *et al.*, 2018).

These silkworms have recently been reported to exhibit various properties such as Alzheimer's prevention, blood sugar lowering, and cholesterol lowering. Thus, they have attracted attention as raw material for healthy functional foods (Ryu *et al.*, 1997; Ahn *et al.*, 2013; Ji *et al.*, 2016). In addition, various functionalities have been scientifically proven to activate functional sericulture through the development of high marketable products and continuous consumption increase (Ji *et al.*, 2016). Furthermore, functional differences have been reported between different

[†]These authors contributed equally.

***Corresponding author.**
Iksoo Kim
Department of Applied Biology, College of Agriculture & Life Sciences, Chonnam National University, Gwangju, 61186, Republic of Korea
Tel: +82-62-530-5117 / FAX: +82-62-530-2069
E-mail: ikkim81@chonnam.ac.kr

varieties of silkworms that are cultivated in farms; thus, there is a need to develop techniques to identify different varieties for industrial use (Park *et al.*, 2021).

Studies in different organisms using genetic markers based on DNA polymorphisms are being conducted to analyze their origin, breed formation, genetic diversity, and relationship with other breeds (Groeneveld *et al.*, 2010). Molecular markers, such as restriction fragment length polymorphism (RFLP) and single nucleotide polymorphism (SNP), are being widely used in plant and animal research and breeding projects. RFLP is based on different length DNA fragments produced after digestion by restriction enzyme due to different location of restriction enzyme sites, and SNP is based on variation at a single position due to a mutation in a single base. SNP is the most widely used molecular marker because they have the advantage of allele mutation, ultra-high throughput, and automation technology (Son *et al.*, 2017). In particular, the SNPs from mitochondrial genome (mitogenome) have been proven to be useful to distinguish several silkworm varieties preserved in Korea (Park *et al.*, 2022). Therefore, in this study, the mitogenomes of ten government-recommended silkworm varieties were analyzed and variety-specific SNPs were selected. In addition, we analyzed the discriminability of major recommended silkworm varieties and explored a method for using them as markers

Materials and Methods

Silkworms

The government-recommended silkworm varieties used in this experiment were DaeHwang (Jam 323X324), JooHwang (Jam 325X326), GoldenSilk (Jam 311X312), DaeBaek (Jam 157X162), HanSaeng (Jam 153X154), DaeBak (Jam 155X156), KumKang (Jam 159X160), KumOk (Jam 125X140), BaekOk (Jam 123X124), and BaekHwang (Jam 327X328). We used breeding standards recommended by the Ministry of Agriculture and Biology, Rural Development Administration (temperature, 24–27°C; humidity, 70%). The silkworms were fed 90% fresh mulberry leaves and bred at 25°C and 16L8D conditions.

Genomic DNA extraction

The genomic DNA of the domesticated silkworms was extracted from five third instar larvae of each of the 10 varieties, pulverizing them using liquid nitrogen and a mortar, and then, using a genomic DNA extraction kit (Qiagen, Hilden, Germany). The extracted DNAs were analyzed for their suitability for sequence analysis by electrophoresis and absorbance analysis using Dropsense96 (Trinean, Gentbrugge, Belgium) and Picogreen (Invitrogen, Oregon, USA).

Mitochondrial genome sequencing and SNP analysis

Each one larva of the ten varieties were amplified for three long overlapping fragments (LFs; COI-ND4, ND5-lrRNA, and lrRNA-COI) using the primers reported by Kim *et al.* (2012). The LFs were amplified using LA Taq™ (Takara Biomedical, Tokyo, Japan) under the following conditions: 96°C for 2 min; 30 cycles at 98°C for 10 s. and 48°C for 15 min; and a final extension step at 72°C for 10 min. The three amplified long fragments were sequenced using the Illumina MiSeq platform (Illumina, San Diego, CA), following the barcode-tagged sequencing technology (Celemics Inc., Seoul, Korea). Each mitogenome was constructed using MITObim ver. 1.9 (Hahn *et al.*, 2013) by *de novo* assembly with one conspecies as reference (GenBank accession number OK358633; Kim *et al.*, 2022). An alignment of mitogenome sequences were performed using MAFFT ver. 6 (Katoh *et al.*, 2002). The SNPs specific to each variety were selected through visual observation.

Primer preparation for discriminant marker region

For SNP verification, a tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) was used according to the method of Medrano and Oliveira (2014). T-ARMS-PCR determines SNPs by one PCR using four primers, two external primers (Outer primer; outer forward (OF) and outer reverse OR), and two inner primers (Inner primer; inner forward (IF) and inner reverse (IR)) to amplify the allele-specific fragment followed by electrophoresis. The inner primer is designed specific to one allele of SNP by modification at 3'-end and binds specifically to that allele but not to the other allele. By placing the outer primers at different distances to each other, the two alleles produce fragments of different sizes. Different primer types were prepared to enable visual identification during electrophoresis (Table 1).

SNP typing

PCR for SNP typing was performed using AccuPower® PCR PreMix (Bioneer, Daejeon, Korea) under the following

Table1. Sequence of T-ARMS-PCR primers used for the identification of silkworm varieties.

SNP	SNP site	Primer sequence (5'→3')	Size (bp)	Description
COIII-5111	C/T	DB-OF: CAGGAGCAATTGGAGTATTAAC	401	
		DB-OR: TATCAATGAGTGATGAGCT		
		DB-IF: TTGAAATTGGAAGAATATGACCY**T**CT	128	etc
		DB-IR: TGGATTAAATGGTGTAATTCTT**C**A**G**	323	DaeBak
COI-2654	A/G	GS-OF: TAATTATTGCTGTACCAACAGG	593	
		GS-OR: TTCATTATATGAATGTTCTGC		
		GS-IF: AGGAGCAGTATTTGCAATTATTG**A**G**A**	373	GoldenSilk
		GS-IR: TAAATAAAGGATATCAGTTAATAAA**G**C**C**	273	etc
COII-3159	T/C	KK-OF: TGAGAATCAATAATTAACCAACG	608	
		KK-OR: ATCTACATCTAATAGTCGGAATTC		
		KK-IF: TAGAACAAATTATTTTTTTT**G**A**C**	368	etc
		KK-IR: AATTAAAATAATTAATGTGTGA**C**C**A**	287	KumKang
ND1-12349	G/A	DH-OF: CGATAACGAGGTAAAGTACC	429	
		DH-OR: TGGTTTACGTTCTGTAGCTC		
		DH-IF: AAATCAAACTATTTCTTGATAT**G**A**A**	130	etc
		DH-IR: ATGTTTTGATATTTATTTTTTT**C**A**C**	348	DaeHwang
COII-3260	A/G	BH-OF: TTTTTTTAGAAATGGCAACATGATCA	433	
		BH-OR: CGATTATCTACATCTAATAGTC		
		BH-IF: AATCGATTTTTATTAG**C**A**G**	269	etc
		BH-IR: CAAATTAATTCAATTATTTG**G**C**T**	205	BaekHwang
ND4-9178	C/T	BO-OF: TAATTTTAATATAATACCAGC	733	
		BO-OR: TTTCAATAATTCGTAGTCATGG		
		BO-IF: GTATTTAAATCATTAAAAATATA**G**A**T**	555	etc
		BO-IR: ACCTTTATTAATAGGAA**C**T**G**	223	BaekOk

*Bold letters: SNP site for the identification of varieties.
**Underlined bold letters: additional substitution to increase amplification efficiency.

conditions: initial denaturation for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, with a subsequent final 7 min extension at 72°C. The amplified products were analyzed on 2% agarose gels.

Results and Discussion

Mitochondrial genome analysis

All mitogenomes are composed of typical gene sets (2 rRNAs, 22 tRNAs, and 13 PCGs) and the A + T-rich region (Table 2; Kim *et al.*, 2022). The sizes of the mitogenomes ranged from 15,637 bp (BaekOk) to 15,682 bp (DaeBak and HanSaeng). Although the lengths of PCGs (3,733 amino acids) and srRNA (781 bp) were identical, lrRNA (1,377–1,378 bp), tRNAs (1,477–1,479 bp), and the A + T- rich region (493–495 bp) were slightly variable (GenBank acc. nos. ON310868–ON310877). A major difference in whole mitogenome size among varieties stemmed from the number of microsatellite-like TA repeats located in the intergenic spacer regions at ND3 and trnA junction (30–81 bp) and trnH and ND4 junction (51–61 bp). Gene arrangements were all identical to other *B. mori* strains reported (Kim *et al.*, 2022).

Table 2. Characteristics of the mitochondrial genomes of the ten silkworm varieties.

Taxon/Strain	Size (bp)	A/T content (%)	PCG		srRNA		lrRNA		tRNA		A+T-rich region		GenBank accession no.
			No. codons[a]	AT (%)	Size (bp)	AT (%)	Size (bp)	AT (%)	Size (bp)	AT (%)	Size (bp)	AT (%)	
Bombyx mori													
BaekHwang	15,677	81.39	3,733	79.61	781	85.53	1,377	84.39	1,477	81.72	494	95.55	ON310868
BaekOk	15,637	81.33	3,733	79.60	781	85.53	1,378	84.40	1,477	81.72	494	95.55	ON310869
DaeBaek	15,661	81.37	3,733	79.61	781	85.53	1,378	84.40	1,477	81.72	494	95.55	ON310870
DaeBak	15,682	81.36	3,733	79.56	781	85.53	1,378	84.47	1,478	81.60	494	95.55	ON310871
DaehWang	15,660	81.34	3,733	79.61	781	85.40	1,378	84.40	1,478	81.73	493	95.33	ON310872
Goldensilk	15,696	81.42	3,733	79.64	781	85.53	1,377	84.39	1,479	81.68	493	95.54	ON310873
HanSaeng	15,682	81.40	3,733	79.62	781	85.53	1,377	84.39	1,477	81.72	495	95.35	ON310874
JoohWang	15,659	81.35	3,733	79.61	781	85.53	1,378	84.40	1,477	81.72	494	95.34	ON310875
KumKang	15,649	81.35	3,733	79.61	781	85.53	1,378	84.40	1,477	81.79	494	95.14	ON310876
KumOk	15,655	81.36	3,733	79.61	781	85.53	1,378	84.40	1,477	81.72	494	95.55	ON310877

[a]Termination codons were excluded in total codon count.

The ten varieties use identical start codon, stop codon, and anticodon.

Single nucleotide polymorphisms

By comparing the mitochondrial genome sequences of the ten varieties, SNPs, which are capable of distinguishing the target variety from the remaining ones, were confirmed in a total of 34 regions, which are located mainly in genic regions (30 among 34 SNPs). The number of variety-specific SNPs in each variety is 13 in DaeBak, 8 in GoldenSilk, 9 in KumKang, 2 in BaekHwang, 1 in BaekOk, and 1 in DaeHwang, and most of the SNP variation types were transition mutations (15 G↔A and 17 C↔T), and only two were transversion mutations (A↔C and A↔T) (data not shown). However, only six varieties (DaeBak, GoldenSilk, KumKang, BaekHwang, BaekOk, and DaeHwang) out of 10 were found to have variety-specific SNPs.

Primer preparation for Tetra-ARMS PCR

To verify the variety-specific SNP, each one SNP was selected for each variety and a primer for performing T-ARMS-PCR was prepared (Table 1) and the sequence alignment of the SNPs is presented in Fig. 1. To identify DaeBak, primer for the 5,111th T►C mutation in the COIII gene was prepared based on tRNA-Ile, and to identify GoldenSilk, primer specific to the 2,654th G►A mutation present in the COI gene was prepared. For KumKang discrimination, the 3,159th C►T mutation in the COII gene was used, and for DaeHwang discrimination, the 12,349th A►G mutation in the NDI gene was used. For the identification of BaekHwang, PCR primers were prepared for the 3,260th G►A mutation in the COII gene and the 9,178th T►C mutation in the ND4 gene for BaekOk identification (Table 1).

Variety identification through Tetra-ARMS PCR

PCR was performed on 10 varieties using the variety-specific primer (Table 1). DaeBak can be identified by amplifying COIII-5,111 SNP: 323-bp long band was formed by DaeBak out of the total 401 bp, whereas a 128-bp long band was formed by the other varieties (Fig. 2A). GoldenSilk can be identified by amplifying COI-2,654 SNP: 373-bp long band was formed only by GoldenSilk out of a total of 593 bp, whereas 273 bp-long band was formed by the other varieties, making it possible to distinguish between GoldenSilk and other varieties (Fig. 2B). When COII-3,159 SNP was amplified, 287-bp long band was formed only by KumKang out of a total of 608 bp, whereas 368-bp long band was formed by other varieties (Fig. 2C). When ND1-12,349 SNP was amplified, 348-bp long band was formed by DaeHwang out of a total of 429 bp, whereas a band of 130 bp

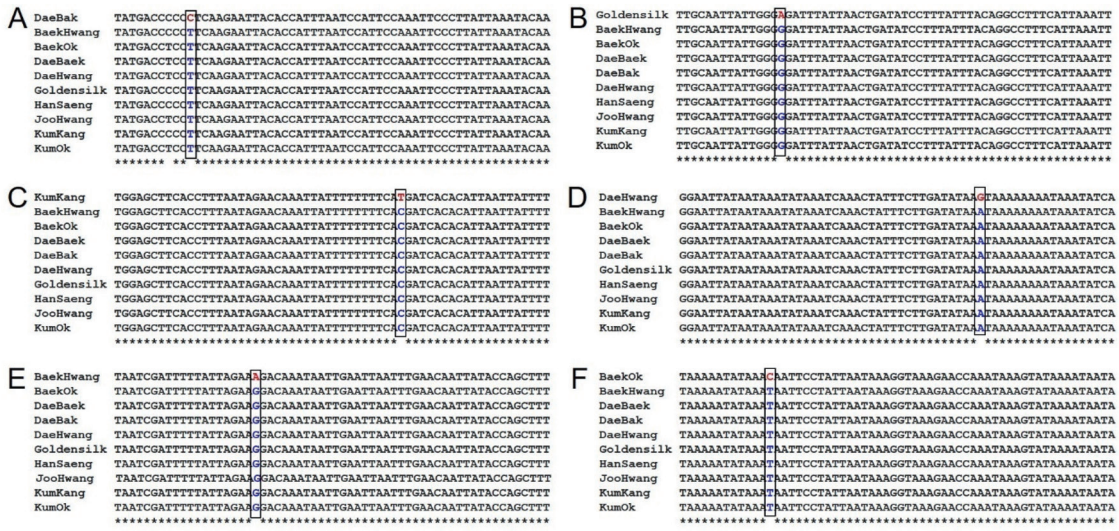

Fig. 1. Sequence alignments of the SNP regions, which were validated by Tetra-ARMS PCR. (A) COIII-5111 for DaeBak, (B) COI-2654 for GoldenSilk, (C) COII-3159 for KumKang, (D) ND1-12349 for DaeHwang, (E) COII-3260 for BaekHwang, and (F) ND4-9178 for BaekOk.

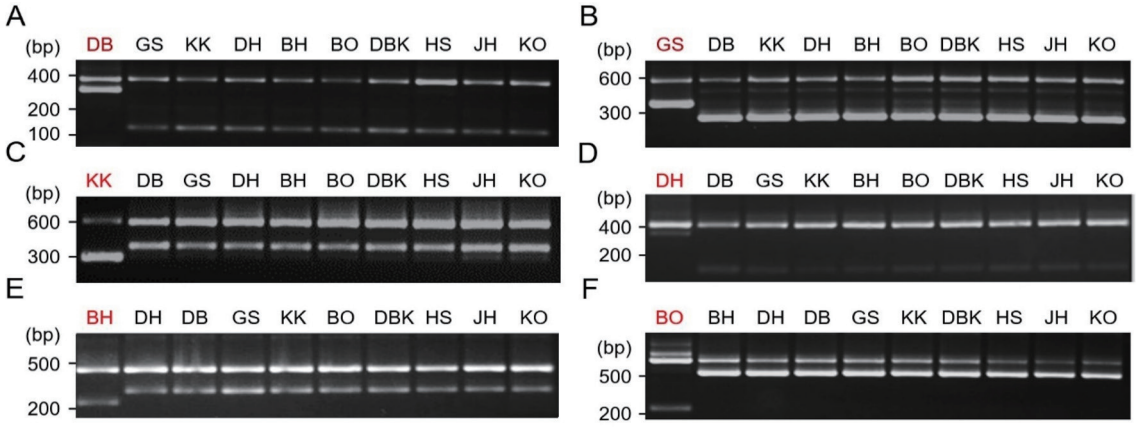

Fig. 2. SNP region tetra-primer ARMS PCR results for variety classification. (A) COIII-5111 for DaeBak, (B) COI-2654 for GoldenSilk, (C) COII-3159 for KumKang, (D) ND1-12349 for DaeHwang, (E) COII-3260 for BaekHwang, and (F) ND4-9178 for BaekOk. DB, DaeBak; GS, GoldenSilk; KK, KumKang; DH, DaeHwang; BH, BaekHwang; BO, BaekOk; DBK, DaeBaek; HS, HanSaeng; JH, JooHwang; KO, KumOk.

was formed in other varieties (Fig. 2D). Among the total 433 bp of COII-3,260 SNP for BaekHwang classification, BaekHwang showed 205-bp long band and other varieties band of 433 bp (Fig. 2E). In the case of ND4-9,178 SNP BaekOk formed a band at 223 bp, whereas other varieties formed a band at 555 bp (Fig. 2F). These primers were further validated using four additional individuals from each variety (data not shown).

It is possible to accurately identify six out of the ten government recommended silkworm varieties using the mitochondrial genome SNPs. Unfortunately, government-recommended silkworm varieties are hybrids, not purebreds, and have mixed traits, making it difficult to judge all the varieties using only the mitochondrial genome SNPs. In addition, since the mitochondrial genome is maternally inherited, it is judged that there is a limit to discriminating the variety using only the SNP of the mitogenome (Kowalczyk *et al*. 2021). Nevertheless, BaekOk and GoldenSilk account for 97% of silkworms produced in Korea,it is worthwhile to be able to discriminate the two variety specific SNPs using T-ARMS-PCR. However, the six varieties identified in this study, it is judged that a whole genome SNP-based variety identification study should be additionally performed to identify all varieties.

Acknowledgements

This study was supported by the 2022 RDA fellowship program of National Institute of Agricultural Science and was supported by a grant (No. PJ01510703) from the Rural Development Administration, Republic of Korea.

References

Ahn HY, Cha JY, Park KR, Kim YR, Cho YS (2013) Improvement effect of fermented silkworm (*Bombyx mori* L.) powder against orotic acid-induced fatty liver in rats. J Life Sci 23, 789-795.

Groeneveld LF, Lenstra JA, Eding H, Toro MA, Scherf B, Pilling D, *et al.* (2010) The genetic diversity in farm animals-a review. Anim Genet 41, 6-31.

Hahn C, Bachmann L, Chevreux B (2013) Reconstructing mitochondria genomes mapping approach. Nucleic Acids Res 41, e129-e129.

Ji SD, Kim NS, Kweon HY, Choi BH, Kim KY, Koh YH (2016) Nutrition composition differences among steamed and freeze-dried mature silkworm larval powders made from 3 *Bombyx mori* varieties weaving different colored cocoons. Int J Indust Entomol 33, 6-14.

Kang PD, Jung IY, Kim KY, Kim MJ, Sohn BH, Lee KG (2010) Breeding of new silkworm variety with peculiar laval mark "Eolrukmal, Hukpyobeom". Int J Indust Entomol 20, 115-116.

Kang PD, Kim KM, Sohn BH, Woo SO, Ryu KS (2003) Breeding of a new silkworm variety, Chugangjam, with a sex-limited larval marking and high silk yielding for summer-autumn rearing season. Int J Indust Entomol 6, 57-61.

Kang PD, Lee SU, Jung IH, Shon BH, Kim YS, Kim KY, *et al.* (2007) Breeding of new silkworm variety golden silk, a yellow cocoon color for spring rearing season. Korean J Seric Sci 49, 14-17.

Katoh K, Misawa K, Kuma KI, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30, 3059-3066.

Kim SW, Kim KY, Kim SR, Kim SB, Ji SD, Kim NS, *et al.* (2018) Breeding of new silkworm variety, 'Chilseongjam' with peculiar larval mark. Int J Indust Entomol 37, 69-72.

Kim JS, Park JS, Kim MJ, Kang PD, Kim SG, Jin BR, *et al.* (2012) Complete nucleotide sequence and organization of the mitochondrial genome of eri-silkworm, *Samia cynthia ricini* (Lepidoptera: Saturniidae). J Asia-Pac Entomol 15, 162-173.

Kim MJ, Park JS, Kim H, Kim SR, Kim SW, Kim KY, *et al.* (2022) Phylogeographic relationships among *Bombyx mandarina* (Lepidoptera: Bombycidae) populations and their relationships to *B. mori* inferred from mitochondrial genomes. Biology 11, 68.

Kowalczyk M, Staniszewski A, Kamiñska K, Domaradzki P, Horecka B (2021) Advantages, possibilities, and limitations of mitochondrial DNA analysis in molecular identification. Folia Biologica 69, 101-111.

Lee SP, Hong KW, Kim KM, Sohn KW (1985) The present situation and improvement trend of silkworm breeding in Korea. Korean J Breed Sci 17, 295-305.

Medrano RFV, Oliveira CA (2014) Guidelines for the tetra-primer ARMS–PCR technique development. Mol Biotechnol 56, 599-608.

Park JW, Lee CH, Jeong CY, Kang SK, Ju WT, Kim SW, *et al.* (2021) Comparison of antioxidant activity according to silkworm cultivars. J Life Sci 31, 1010-1018.

Park JS, Kim MJ, Kim SW, Kim KY, Kim SR, Kim I (2022) Molecular identification of the strains of the domestic silkworm, *Bombyx mori* (Lepidoptera: Bombycidae), which are endemic to Korea, based on single nucleotide polymorphisms in mitochondrial genome sequences a, J Asia-Pac Entomol 25, 101922.

Ryu KS, Lee HS, Chung SH, Kang PD (1997) An activity of lowering blood-glucose levels according to preparative conditions of silkworm powder. Kor J Seric Sci 39, 79-85.

Ryu KS, Lee HS, Kim I (2002) Effects and mechanisms of silkworm powder as a blood glucose-lowering agent. Int J Indust Entomol 4, 93-100.

Ryu KS, Kim I, Ahn MY, Lee HS, Kim JW, Lee P (2003) Functionality research on silkworm and sericultural products. Food Sci Indust. 36, 15-24.

Son JH, Cheong YK, Park JC, Kim YK, Kim KH, Park TI, *et al.* (2017) Construction of complete DNA marker set for 32 Korean wheat cultivar identification. J Korean Soc Int Agric 29, 150-154.