Korean J. Plant Res. 26(3):397-402(2013)
http://dx.doi.org/10.7732/kjpr.2013.26.3.397

Print ISSN 1226-3591
Online ISSN 2287-8203

Original Research Article

Molecular Authentication of Morus Folium Using Mitochondrial *nad*7 Intron 2 Region

Chi-Gyu Jin, Min-Kyeung Kim, Jin-Young Kim, Myung-Suk Sun, Woo-Saeng Kwon and Deok-Chun Yang*

Department of Oriental Medicinal Materials and Processing, Kyung Hee University, Suwon 449-701, Korea

Abstract - Morus Folium (Sang-yeop in Korean) is one of the most important Oriental medicinal plants. In Korea, both *M. alba* and *M. cathayana* are regarded as the botanical sources for Morus Folium. In order to discriminate *M. alba* and *M. cathayana* from their adulterant, *M. tricuspidata*, mitochondrial NADH dehydrogenase subunit 7 (*nad*7) intron 2 region was targeted for molecular analysis with universal primers. DNA polymorphisms, including SNP sites, insertions, and deletions, were detected among these three species sequencing data. Based on these DNA polymorphisms, specific primers were designed for the three species respectively. Multiplex PCR was conducted for molecular authentication of *M. alba*, *M. cathayana*, and *M. tricuspidata* with specific primers. The present results indicate that it is possible to identify Morus Folium from its adulterant using mitochondrial *nad*7 intron 2 region. The established multiplex-PCR system was proved to be effective for identification of Morus Folium. The results indicate that mitochondrial introns can be used for inter-specific polymorphic study, and the described method can be applied for molecular identification of medicinal materials.

Key words - Morus Folium; *M. alba*; *M. cathayana*; *M. tricuspidata*; *nad*7 intron; Polymorphism; Multiplex PCR

Introduction

Morus Folium (Sang-yeop in Korean), the dried leaf of *Morus alba* Line, is one of the most important Oriental medicinal plants used in the Orient. Plants of the *Morus* genus are known to be rich in flavonoids and have been shown to exhibit anti-HIV, anti-oxidative, antihypotensive and cytotoxic activities (Nomura *et al*., 1980; Nomura *et al*., 1978; Luo *et al*., 1995; Hosseinzadeh *et al*., 1999; Kim *et al*., 1999; Doi *et al*., 2001). Morus Folium has been traditionally used in Korea, China and Japan for various medicinal purposes, such as clear the lung and moisten dryness, clear the liver, promote hair, eliminate abiding blood, stop thirst, soothe and disperse wind-heat, aching and numbness of joints, moisten dryness (Chen *et al*., 1995).

In Korea, Morus L. species ca. 11 (4, with 1 cultivated) (Park, 2007) especially both *M. alba* Line and *M. cathayana* are regarded as the botanical sources for Morus Folium. However, the leaves of *M. tricuspidata* are always misidentified as Morus Folium due to their morphological similarities, especially the Morus Folium products in the market which are packaged in forms of powers of slices. Traditional authentication methods, which have relied on morphological and histological differences, are limited and quite often unreliable. Chemical analysis via examination of secondary metabolites is another ways to tried, but the metabolite profiles are significantly affected by environmental growth conditions as well as storage conditions (Yip *et al*., 1985). In comparison, DNA analysis by molecular techniques is highly accepted for the proper identification of medicinal plants, because the identification of medicinal plants based on genotype is not influenced by growth stage and environmental conditions of plants (Zhu *et al*., 2004). Several molecular biological methods such as random amplified polymorphic DNA (RAPD) (Cui *et al*., 2003; Shim *et al*., 2003; Shaw and But, 1995), arbitrarily primed polymerase chain reaction (AP-PCR) (Cheung *et al*., 1994), sequence characterized-amplified region (SCAR) (Choi *et al*., 2008; Wang *et al*., 2001), restriction fragment length polymorphism (RFLP) (Ngan *et al*., 1999), loop-mediated isothermal amplification

*Corresponding author. E-mail : dcyang@khu.ac.kr

(LAMP) (Sasaki *et al*., 2008), amplification fragment length polymorphism (AFLP) (Ha *et al*., 2002) and DNA microarray (Zhu *et al*., 2008) have been described until now. In this study, we developed a DNA technique to discriminate *M. alba* and *M. cathayan* from *M. tricuspidata,* by analyzing the mitochondrial *nad*7 intron 2 region.

Materials and Methods

Plant Materials

Plant samples of *M. alba*, *M. cathayan*, and *M. tricuspidata* were collected from Korea local market, Plant DNA Bank and The Wild Plant Seed Bank (http://seedbank.pdrc.re.kr/), respectively (Table 1). Three *Morus* standard samples were collected from Plant DNA BanK (http://pdbk.korea.ac.kr/), and The Wild Plant Seed Bank (http://seedbank.pdrc. re.kr/). Each standard sample number is *M. alba* (DbHN1489), *M. cathayan*(KRIBBSD2333), and *M. tricuspidata*(KRIBBSD2334).

DNA extraction and PCR amplification of *nad*7 intron 2 region

The collected sample leaves were frozen in liquid nitrogen and ground into fine powder. Genomic DNA was isolated and purified using a Plant DNA extraction kit (Gene All, General bio system, Seoul, Korea). The primer pairs used for amplification of *nad7* intron2 region were *nad*7/2 (5'-GCTTTACCTTATTCTGATCG-3') and *nad*7/3 (5'-TGTTCTTGGGCCATCATAGA-3') (Duminil et al., 2002). PCR amplification was performed in a total volume of 20 μl, and the reaction mixture consisted of each of the primers at a concentration of 0.5 μM, 50 ng of template DNA, and 10 μl of 2 × PCR premix (Genotech, South Korea). The amplification profile consisted of 1 pre-denaturation cycle of 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, 1 min at 72°C, and a final extension at 72°C for 7 min. The PCR products were migrated on a 1.0% agarose gel electrophoresis and detected by ethidium bromide staining under UV.

Sequencing and DNA sequence analysis

The PCR products were purified used PCR product purification kit (GENEALL PCR SV, General bio system), according to the manufacturer's instructions. The purified products were sequenced by Genotech, Inc. The DNA sequences of the mitochondrial *nad*7 intron2 regions obtained in sequencing experiments were compiled using SeqMan software, and the sequences were edited with BioEdit program (Hall *et al*., 1999). Multiple sequence alignments

Table 1. Plant samples used in this study

Species	Voucher	Collection Date	GenBank accessions of *nad*7 intron2
M. alba	DbHN1489	2010.3	
M. alba	MAK301	2010.3	
M. alba	MAK302	2010.3	
M. alba	MAK303	2010.4	JF340231
M. alba	MAK304	2010.3	
M. alba	MAK305	2010.3	
M. alba	MAK306	2010.4	
M.cathayana	KRIBBSD2333	2010.4	
M.cathayana	MCK201	2010.3	JF340232
M.cathayana	MCK202	2010.3	
M.cathayana	MCK203	2010.3	
M.tricuspidata	KRIBBSD2334	2010.3	
M.tricuspidata	MTK101	2010.3	JF340233
M.tricuspidata	MTK102	2010.4	
M.tricuspidata	MTK103	2010.4	

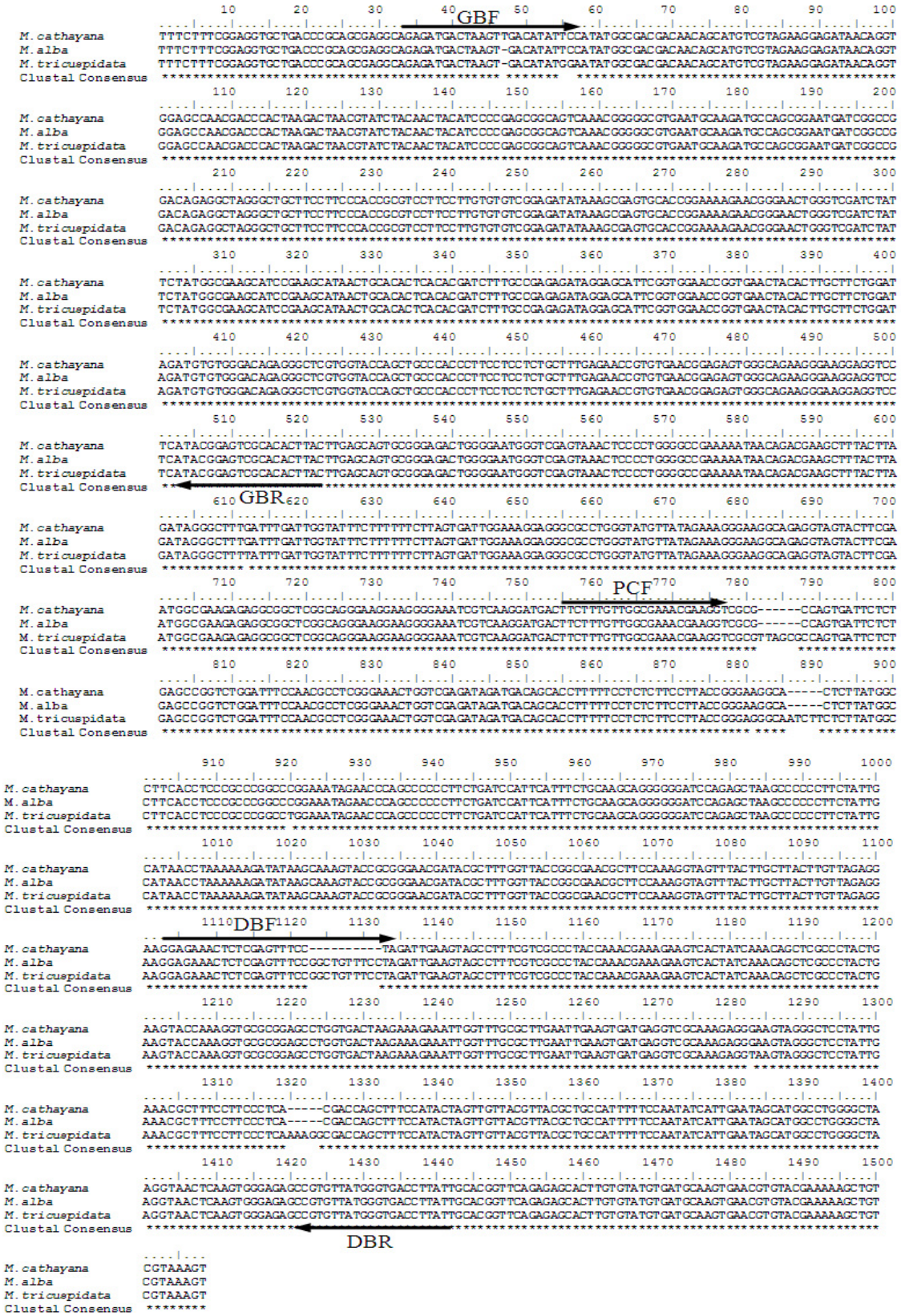

Fig. 1. Comparison of the *nad*7 intron 2 regions of *M. cathayana, M. alba*, and *M. tricuspidata.*

were performed using online ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/).

Design of specific primers

Specific primers were designed for *M. alba, M. cathayana,* and *M. tricuspidata*, respectively, on the basis of the DNA polymorphisms detected (Fig. 1). Primers GBF and GBR were designed for authentication of *M. tricuspidata* based on its specific SNP sites, while primers DBF and DBR were designed for specific identification of *M. cathayana*, based on its specific 10 bp-deletion. Primer PCF, another sense primer of DBR, was designed to provide a positive control for all samples. The sequences and orientations of specific primers and common primers were shown in Table 2 and Fig. 2, respectively.

Multiplex PCR

Molecular authentication of Morus Folium was performed using multiplex PCR. Five primers, PCF, DBF, DBR, GBF and GBR, were used simultaneously in multiplex PCR amplification. The reaction mixture was identical with described above except the concentration of primers: the concentrations of five primers, PCF, DBF, DBR, GBF and GBR, were 0.7 μM, 0.25 μM, 0.38 μM, 0.38 μM and 0.38 μM, respectively. PCR amplification was performed in a total volume of 20 μl. The amplification profile consisted of 1 pre-denaturation cycle of 4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 65°C, 1 min at 72°C, and a final extension at 72°C for 7 min. The PCR products were migrated on a 1.0% agarose gel.

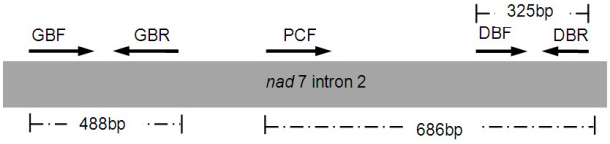

Fig. 2. Schematic diagrams of the primers used in multiplex PCR.

Results and Discussion

Nuclear internal transcribed spacer (ITS) region and chloroplast *trn*L-F region have been demonstrated useful for identification at the species level (Baldwin 1992; Quandt and Stech, 2004). However, these two regions cannot be used for authentication of Morus Folium due to their less polymorphism. In comparison, mitochondrial genome has a remarkable feature of slow-rate evolution (Wolfe *et al*., 1987), resulted in low rate of sequence change. However, the sequence of introns and intergenic regions are highly variable due to the lack of sequence conservation in function (Hu and Luo, 2006). The mitochondrial *nad*7 introns have been demonstrated useful for intra-specific polymorphic studies (Wang *et al*., 2009). In this study, the mitochondrial *nad*7 intron 2 region was targeted for molecular analysis and this region was proved to be useful for discrimination of *M. alba, M. cathayana*, and *M. tricuspidata.*

The mitochondrial *nad*7 intron 2 regions of three species were PCR amplified using the universal primer sets of *nad*7 intron 2 region, 'nad7/2F' and 'nad7/3R'. The *nad*7 intron 2 regions of *M. alba, M. cathayana*, and *M. tricuspidata* were determined to be 1491 bp, 1482 bp, and 1507 bp, respectively. Their sequences were registered in GeneBank with the accession numbers JF340231-JF340233.

Table 2. Primers used in this study

Primer name	Nucleotide sequences (5'→3')	Location
*nad*7/2	GCT TTACCTTATTCTGATCG	*nad*7 exon
*nad*7/3	TGTTCTTGGGCCATCATAGA	*nad*7 exon
PCF	TTGTTGGCGAAACGAAGGT	759－777
DBF	GGAGAAACTCTCGAGTTTCCTA	1103-1134
DBR	AATAAGGTCACCCATAACACG	1421-1442
GBF	AGAGATGACTAAGTGACATATGGA	33-58
GBR	GTA AGT GTGCGACT CCGTAT	503-522

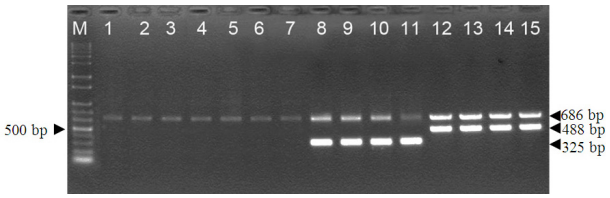

Fig. 3. Gel image of multiplex-PCR products. Lane M: 1,000 bp DNA ladder; lane 1-7: *Morus alba*; lane 8-11: *Morus cathayana*; lane 12-15: *Maclura tricuspidata.*

Multiplex alignments of the *nad*7 intron 2 regions of *M. cathayana*, *M. alba,* and *M. tricuspidata* were perfomed with the CLUSTAL X program. DNA polymorphisms, including SNP sites, insertions, and deletions, were detected among these three species. Based on the three *M. tricuspidata*-specific SNP sites on the 54^{th}-56^{th} positions, primer set GBF and GBR were designed. *M. cathayana*-specific primer set, DBF and DBR, were designed according to the deletion located at $1,123^{th}$-$1,132^{th}$ nucleotide positions. Primer PCF was designed to provide a positive control for all the samples, by combination with primer DBR (Fig. 1).

Molecular discrimination of *M. cathayan*a, *M. alba,* and *M. tricuspidata* was conducted using multiplex PCR with the five primers described above. The combination of five specific primers, as shown in Fig. 3, yielded expected amplicons for different species. The lengths of specific amplicons were determined according the positions of corresponding specific primer sets. All the three species generated a universal band of 686 bp with primer PCF and DBR, which provide a positive control to show that the PCR amplification works properly. As expected, *M. cathayan*a and *M. tricuspidata* yielded their specific amplicons with sizes of 325 bp and 488 bp, which generated by their specific primer sets, GBF-GBR and DBF-DBR, respectively. Therefore, *M. alba*, *M. cathayana,* and *M. tricuspidata* can be clearly differentiated from each other by the developed multiplex PCR system.

In the present study, we identified three *Morus* species by simultaneously amplifying their specific alleles using multiplex PCR. The present results indicate that it is possible to identify Morus Folium from its adulterant using mitochondrial *nad*7 intron 2 regions. Compared with other molecular markers, this method is reliable, time-saving, and can be used for numerous repeated tests of many medicinal plants. We strongly recommend multiplex PCR for identification of medicinal plants, and the methodology presented in this study can be adapted for authentication of other medicinal materials.

Acknowledgements

This research was supported by iPET (112142-05-1-CG000), Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries, Republic of Korea.

Literature Cited

Baldwin, B.G. 1992. Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. Mol. Phylogen. 1:3-6.

Chen, F.J., N. Nakashima, I. Komura, M. Kimura, N. Asano and S. Koya. 1995. Potentiation effects on pilocarpine-induced saliva secretion, by extracts and N-containing sugars derived from mulberry leaves. Biol. Pharm. Bull. 18:1676-1680.

Cheung, K.S., H.S. Kwan, P.P. But and P.C. Shaw. 1994. Pharmacognostical identification of American and Oriental ginseng roots by genomic fingerprinting using arbitrarily primed polymerase chain reaction (AP-PCR). J. Ethnopharmacol. 42:67-69.

Choi, Y.E., C.H. Ahn, B.B. Kim and E.S. Yoon. 2008. Development of species specific AFLP-derived SCAR marker for authentication of Panax japonicus C. A. Meyer. Biol. Pharm. Bull. 31:135-138.

Cui, X.M., C.K. Lo, K.L. Yip, T.T.X. Dong and K.W.K. sim. 2003. Authentication of *Panax notoginseng* by 5S-rRNA Spacer Domain and Random Amplified Polymorphic DNA (RAPD) Analysis. Planta Med. 69:584-586.

Doi, K., T. Kojima, M. Makino, Y. Kimura and Y. Fujimoto. 2001. Studies on the constituents of the leaves of *Morus alba* L. Chem. Pharm. Bull. 49: 151-153.

Duminil, J., M.H. Pemonge and R.J. Petit. 2002. A set of 35 consensus primer pairs amplifying genes and introns of plant mitochondrial DNA. Molecular Ecology Notes 2:428-430.

Ha, W.Y., P.C. Shaw, J. Liu, F.C.F Yau and J. Wang. 2002. Authentication of *Panax ginseng* and *Panax quinquefolius*

using amplified fragment length polymorphism (AFLP) and directed amplification of minisatellite region DNA (DAMD). J. Agric. Food Chem. 50:1871-1875.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analtsis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41:95-98.

Hosseinzadeh, H. and A. Sadeghi. 1999. Antihyperglycemic effects of *Morus nigra* and *Morus alba* in mice. Pharm. Pharmacol. Lett. 9:63-65.

Hu, D and Z. Luo. 2006. Polymorphisms of amplified mitochondrial DNA non-coding regions in *Diospyros* spp. Sci. Horticult. 109:275-281.

Kim, S., J. Gao, W.C. Lee, K. Ryu, K. Lee and Y. Kim. 1999. Antioxidative flavonoids from the leaves of *Morus alba*. Archives of Pharmacal Research 22:81-85.

Luo, S.D., J. Nemec and B.M. Ning 1995. Anti-HIV flavonoids from *Morus alba*, Yunnan Zhiwu Yanjiu 17:89-95.

Ngan, F., P. Shaw, P. But and J. Wang. 1999. Molecular authentication of *Panax* species. Phytochemistry 50:787-791.

Nomura, T and T. Fukai. 1980. Hypotensive constituent, kuwanon H, a new flavones derivative from the root bark of the cultivated mulberry tree (*Morus alba* L.). Heterocycles 14:1943-1951.

Nomura, T., T. Fukai and M. Katayanagi. 1978. Studies on the constituents of the cultivated mulberry tree. III. Isolatin of four new flavones, kuwanon A, B, C, and oxydihydromorusin from the root bark of *Morus alba* L. Chem. Pharm. Bull. 26:1453-1458.

Park, C.h. 2007. The Genera of Vaschlar Plants of Korea, Flora of Korea Editorial Committed 247-249.

Quandt, D., M. Stech. 2004. Molecular evolution of the *trn*-TUGC *trn*FGAA region in Bryophytes. Plant Biol. 6(5):545-554.

Sasaki, Y., K. Komatsu and S. Nagumo. 2008. Rapid detection of *Panax ginseng* by loop-mediated isothermal amplification and its application to authentication of ginseng. Biol. Pharm. Bull. 31:1806-1808.

Shaw, P.C and P.P.H. But. 1995. Authentication of *Panax* species and their adulterants by random-primed polymerase chain reaction. Planta Med. 61:466-469.

Shim, Y.H., J.H. Choi, C.D. Park, C.J. Lim, J.H. Cho and H.J. Kim. 2003. Molecular differentiation of *Panax* species by RAPD analysis. Archives of Pharmacal Research 26:601-605.

Wang, H., H. Sun, W.S. Kwon, H. Jin and D.C. Yang . 2009. Molecular identification of the Korean ginseng cultivar "Chunpoong" using the mitochondrial nad7 intron 4 region. Mitochondrial DNA 20:41-45.

Wang, J., W.Y. Ha, F.N. Ngan, P.P.H. But and P.C. Shaw. 2001. Application of sequence characterized amplified region (SCAR) analysis to authenticate *Panax* species and their adulterants. Planta Med. 67:781-783.

Wolfe, K.H., W.H. Li and P.M. Sharp.1987. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. PNAS 84:9054-9058.

Yip, T.T., C.N. Lau, P.P. But and Y.C. Kong. 1985. Quantitative analysis of ginsenosides in fresh *Panax ginseng*. Am. J. Chin. Med. 13:77-88.

Zhu, S., H. Fushimi and K. Komatsu. 2008. Development of a DNA microarray for authentication of ginseng drugs based on 18S rRNA gene sequence. J. Agricult. Food Chem. 56: 3953-3959.

Zhu, S., H. Fushimi. S. Cai and K. Komatsu. 2004. Species identification from ginseng drugs by multiplex amplification refractory mutation system (MARMS). Planta Med. 70:189-192.

(Received 5 June 2013 ; Revised 19 June 2013 ; Accepted 21 June 2013)