Plant Biotechnol Rep (2010) 4:223–228
DOI 10.1007/s11816-010-0142-3

ORIGINAL ARTICLE

Molecular identification of oriental medicinal plant *Schizonepeta tenuifolia* bunge (Hyung-Gae) by multiplex PCR

Baigalmaa Jigden · Hongtao Wang · Yeon-Ju Kim · Narantuya Samdan · Jun-Gyo In · Deok Chun Yang

Received: 13 January 2009 / Accepted: 9 June 2010 / Published online: 24 June 2010

Abstract *Schizonepeta tenuifolia* (Korean name "Hyung-Gae") is an oriental medicinal plant that is widely used in Korea, China and Japan. *S. tenuifolia* (Hyung-Gae) has many pharmacological activities and is mostly used for many medicinal preparations. The dried aerial part (spikes and stems) of three oriental medicinal plants, *S. tenuifolia* (Hyung-Gae), *Agastache rugosa* (Kwhak-Hyang) and *Elsholtzia ciliata* (Hyang-Yoo) belonging to the same family, mint family Labiaceae, have such similar shape and smell that it is difficult to differentiate between them. The *trn*L-F regions of chloroplast DNA of the three medicinal plants were sequenced and used as targets in multiplex PCR reaction to identify *S. tenuifolia*. After alignment of *trn*L-F sequences of the authenticated plant samples, one single nucleotide polymorphism (SNP) specific to *S. tenuifolia* was found. Based on this SNP, a new primer was designed that specifically amplifies the *trn*L-F region of *S. tenuifolia*. The established multiplex-PCR was proven to be effective in the differentiation of commercial *S. tenuifolia* samples from *A. rugosa* and *E. ciliata*. This rapid and accurate molecular method is highly promising for use in the food industry.

Keywords *Schizonepeta tenuifolia* · *Agastache rugosa* · *Elsholtzia ciliata* · Multiplex PCR · Single nucleotide polymorphism · *trn*L-F

B. Jigden · H. Wang · Y.-J. Kim · J.-G. In · D. C. Yang (✉)
Department of Oriental Medicinal Material and Processing, College of Life Science, Kyung Hee University, 1 Seocheon-Dong, Giheung-gu, Yongin-si, Gyeonggi-do 449-701, South Korea
e-mail: deokchunyang@yahoo.co.kr

N. Samdan
Health Sciences University of Mongolia, Ulaanbaatar, Mongolia

Introduction

Schizonepeta tenuifolia Briquet is an important oriental medicinal plant belonging to the mint family Labiaceae (=Labiatae). This plant is collected in autumn or early winter, dried in shade, and used unprepared or stir-baked to charcoal in Korea, China and Japan for oriental medicinal purposes. The dried aerial part (spikes and stems) of *S. tenuifolia* is called "Hyung-Gae" in Korea and "Jingjie" in Chinese. *S. tenuifolia* (Hyung-Gae) has a slightly "warm" property according to the theory of traditional Chinese medicine (TCM). The major active compounds of Hyung-Gae were found to be volatile oils consisting mainly of menthone, monoterpenic (Yang et al. 2002), schizonepetosides, schizonol, schizonodiol, flavonoids known as diosmetin, hesperetin, and luteolin, and a small amount of D-limonene (Fung 2002; Hu et al. 2005, 2006; Oshima et al. 1989; Yang et al. 2005; Yen 1992).

A wide variety of pharmacological activities of Hyung-Gae have been reported, including diaphoresis, dispelling pathogenic wind-cold and skin eruptions (Chen and Li 1993; Lu et al. 2002), and strong antimicrobial (Chang and But 1986; Zheng et al. 1997), insecticidal, antioxidant (Kirby and Schmidt 1997; Park et al. 2006), and anti-inflammatory activity (Matsuta et al. 1996). *S. tenuifolia* is available in two forms: sun-dried and carbonized. In the process of carbonization, the aerial part of *S. tenuifolia* is stir-fried at a high temperature until its exterior surface becomes blackish-brown and its interior surface turns dark yellow. The carbonized herb, in particular, has hemostatic properties and is a remedy for bloody stool and metrorrhagia (Guo et al. 1997; Zhang et al. 2006; Zhu 1998). In the herbal market, two medicinal plants, *Agastache rugosa* (Korean name 'Kwhak-Hyang') and *Elsholtzia ciliata* (Korean name 'Hyang-Yoo'), are often regarded as adulterants of Hyung-Gae.

The morphological shapes of their aerial parts are so similar to those of *S. tenuifolia* that it is difficult to discriminate *S. tenuifolia* ('Hyung-Gae') from *A. rugosa* ('Kwhak- Hyang') and *E. ciliata* ('Hyang-Yoo').

The authentication of *S. tenuifolia* ('Hyung-Gae') has traditionally relied upon morphological and histological inspections. However, it is often difficult to determine the source of a herb from its morphology, as the majority of herb products are packaged in the form of powders or shredded slices, which no longer bear the original features of the plants, thus rendering their authentication by morphological and histological techniques very difficult, if not impossible. Therefore, the method of DNA analysis, rather than the traditional authentication methods, is clearly desirable. Many molecular biological methods have been used for the DNA analysis of medicinal plants, including restriction fragment length polymorphism (RFLP) (Ngan et al. 1999), randomly amplified polymorphic DNA (RAPD) (Cui et al. 2003; Shim et al. 2003), amplified fragment length polymorphism (AFLP) (Ha et al. 2002), SCAR (Choi et al. 2008; Wang et al. 2001), loop-mediated isothermal amplification (LAMP) (Sasaki et al. 2008), and DNA microarray (Zhu et al. 2008). In this study, we developed a new molecular technique to discriminate *S. tenuifolia* ('Hyung-Gae') from *A. rugosa* ('Kwhak-Hyang') and *E. ciliata* ('Hyang-Yoo'), using multiplex PCR amplifying the *trn*L-F region. It is an easier, more reproducible and robust approach for the authentication and differentiation of *S. tenuifolia* ('Hyung-Gae').

Materials and methods

Plant materials

Thirteen samples of *S. tenuifolia* ('Hyung-Gae') were collected: two actively growing authenticated plants were harvested from field plots in Suwon and Ok-Chun cities in South Korea, seven commercial materials were purchased from local markets in South Korea, and four commercial materials were purchased from local markets in China. Six samples of *A. rugosa* ('Kwhak-Hyang') were collected: two actively growing authenticated plants were harvested from field plots in Suwon and Ok-Chun cities in South Korea, two commercial materials were purchased from local markets in South Korea, and two commercial materials were purchased from local markets in China. Four samples of *E. ciliata* ('Hyang-Yoo') were collected: one actively growing authenticated plant was harvested from a field plot in Suwon city in South Korea, two commercial materials were purchased from local markets in South Korea, and one commercial material was purchased from a local market in China (Table 1).

DNA extraction and PCR of *trn*L-F region

The collected samples were ground in liquid nitrogen and their DNA was isolated from the ground samples using a Plant DNA isolation mini kit (GeneALL; General Biosystem, Korea). The oligonucleotide primers for the amplification of the *trn*L-F region were synthesized by Genotec (Daejeon, Korea). The universal primers annealed at the 5′ and 3′ ends of the *trn*L-F region were '*trn*L-Fc' (5′-CGA AAT CGG TAG ACG CTA CG-3′) and '*trn*L-Ff' (5′-ATT TGA ACT GGT GAC ACG AG-3′), respectively (Taberlet et al. 1991). The PCR amplification (ASTEC Korea, pc 802010) was conducted in a 20-μl final reaction volume with 2× *Taq* pre-mix I (SolGent), 10 μl of each 5-pmol primer and 50 ng of extracted plant DNA. The PCR was conducted for 36 cycles, with a thermal profile as follows: pre-denaturation at 96°C for 2 min, denaturation at 96°C for 30 s, primer annealing at 50°C for 30 s, and extension at 72°C for 90 s, The final cycle included a extension at 72°C for 7 min, in order to ensure full extension of the products.

Gel electrophoresis and DNA sequencing

The PCR products were analyzed via the electrophoresis (Mupid-exU) of an 8.5-μl aliquot through a 1.0% (wt/vol) agarose gel (Agarose, CALEDON), stained with ethidium bromide (0.5 μl/ml), and visualized via UV trans-illumination. A 1-kb ladder (Bioneer, Daejeon, Korea) was employed as a size marker. All the PCR product was purified with a PCR product purification kit (GeneALL PCR SV; General Biosystem), in accordance with the manufacturer's instructions and sequenced by Genotec, in Daejeon, South Korea.

*trn*L-F sequence comparison

After sequencing, the full sequences of the *trn*L-F regions were compiled with SeqMan software. The *trn*L-F sequences of the related taxa were obtained from GenBank. All the *trn*L-F sequences were edited using the BioEdit program (Hall 1999). Multiple alignments were performed with the CLUSTAL X program (Thompson et al. 1997).

Design of specific primers

Among the single nucleotide polymorphisms (SNPs) detected, SNPs that were specific for *S. tenuifolia* (Hyung-Gae), *A. rugosa* (Kwhak-Hyang) and *E. ciliata* (Hyang-Yoo) were selected, respectively (Fig. 2). The sequences of specific primers (HG5, KW5, HY5) are 5′-TTT ATTT TTA CGT TGA AGA AAA ATG-3′; 5′-CAA AAA ACG AAA AAA TA-3′, and 5′-ATT CTT TGA CAA ACT GGG

Table 1 The list of samples used in this study

No.	Sample name	Scientific name	Place	City	Nation
1	Hyung-Gae 01	*Schizonepeta tenuifolia*	National Institute Crop Science	Su-Won	Korea
2	Hyung-Gae 02	*Schizonepeta tenuifolia*	Kyung-dong market	Seoul	Korea
3	Hyung-Gae 03	*Schizonepeta tenuifolia*	Local market	An-Kook	China
4	Hyung-Gae 04	*Schizonepeta tenuifolia*	Local market	Dae-Gu	Korea
5	Hyung-Gae 05	*Schizonepeta tenuifolia*	Local market	Ham-Yang	Korea
6	Hyung-Gae 06	*Schizonepeta tenuifolia*	Kyung-dong market	Seoul	Korea
7	Hyung-Gae 07	*Schizonepeta tenuifolia*	Local market	Sung-Do	China
8	Hyung-Gae 08	*Schizonepeta tenuifolia*	Local market	An-Kook	China
9	Hyung-Gae 09	*Schizonepeta tenuifolia*	Local market	San-Dong	China
10	Hyung-Gae 10	*Schizonepeta tenuifolia*	Local market	Chang-Won	Korea
11	Hyung-Gae 11	*Schizonepeta tenuifolia*	Local market	Kuem-San	Korea
12	Hyung-Gae 12	*Schizonepeta tenuifolia*	National Institute Crop Science	Ok-Cheon	Korea
13	Hyung-Gae 13	*Schizonepeta tenuifolia*	Kyung-dong market	Seoul	Korea
14	Kwhak-Hyang 01	*Agastache rugosa*	National Institute Crop Science	Seoul	Korea
15	Kwhak-Hyang 02	*Agastache rugosa*	National Institute Crop Science	Ok-Cheon	Korea
16	Kwhak-Hyang 03	*Agastache rugosa*	Local market	Dae-Gu	Korea
17	Kwhak-Hyang 04	*Agastache rugosa*	Local market	Sung-Do	China
18	Kwhak-Hyang 05	*Agastache rugosa*	Local market	An-Kook	China
19	Kwhak-Hyang 06	*Agastache rugosa*	Local market	Chang-Won	Korea
20	Hyang-Yoo 01	*Elsholtzia ciliata*	National Institute Crop Science	Ok-Cheon	Korea
21	Hyang-Yoo 02	*Elsholtzia ciliata*	Local market	Dae-Gu	Korea
22	Hyang-Yoo 03	*Elsholtzia ciliata*	Local market	An-Kook	China
23	Hyang-Yoo 04	*Elsholtzia ciliata*	Local market	Kuem-San	Korea

Table 2 Oligonucleotide sequences of primers used for multiplex-PCR

Primer name	Primer sequences (5′–3′)	Reference
*trn*L-Fc	5′-CGA AAT CGG TAG ACG CTA CG-3′	Taberlet et al. (1991)
*trn*L-Ff	5′-ATT TGA ACT GGT GAC ACG AG-3′	Taberlet et al. (1991)
HG5	5′-TTT ATT TTA CGT TGA AGA AAA ATG-3′	Present study
KW5	5′-CAA AAA ACG AAA AAA TA-3′	Present study
HY5	5′-ATT CTT TGA CAA ACT GGG GT-3′	Present study

GT-3′, respectively (Table 2). The oligonucleotide primers were synthesized and prepared by Genotec (Daejeon). The orientation of specific primers and common primers in the *trn*L-F region are shown in (Fig. 1).

Multiplex PCR

Multiplex PCR was conducted with identical concentrations and temperature conditions as described above, except for the following: the concentration of the forward and reverse primers "*trn*L-Fc" was 0.25 μM and of "*trn*L-Ff" was 0.75 μM; the concentration of all four specific primers (HG5, KW5, HY5) was 0.25 μM.

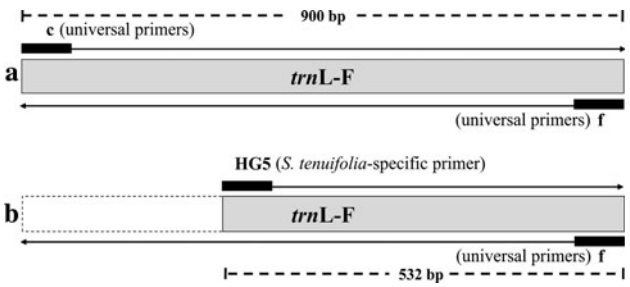

Fig. 1 Schematic diagrams of the PCR-amplified *trn*L-F region of *S. tenuifolia* ('Hyung-Gae'). **a** The PCR product amplified with universal primer sets, '*trn*L-Fc' and '*trn*L-Ff'. **b** The PCR product amplified with *S. tenuifolia* ('Hyung-Gae')-specific primer, 'HG5'

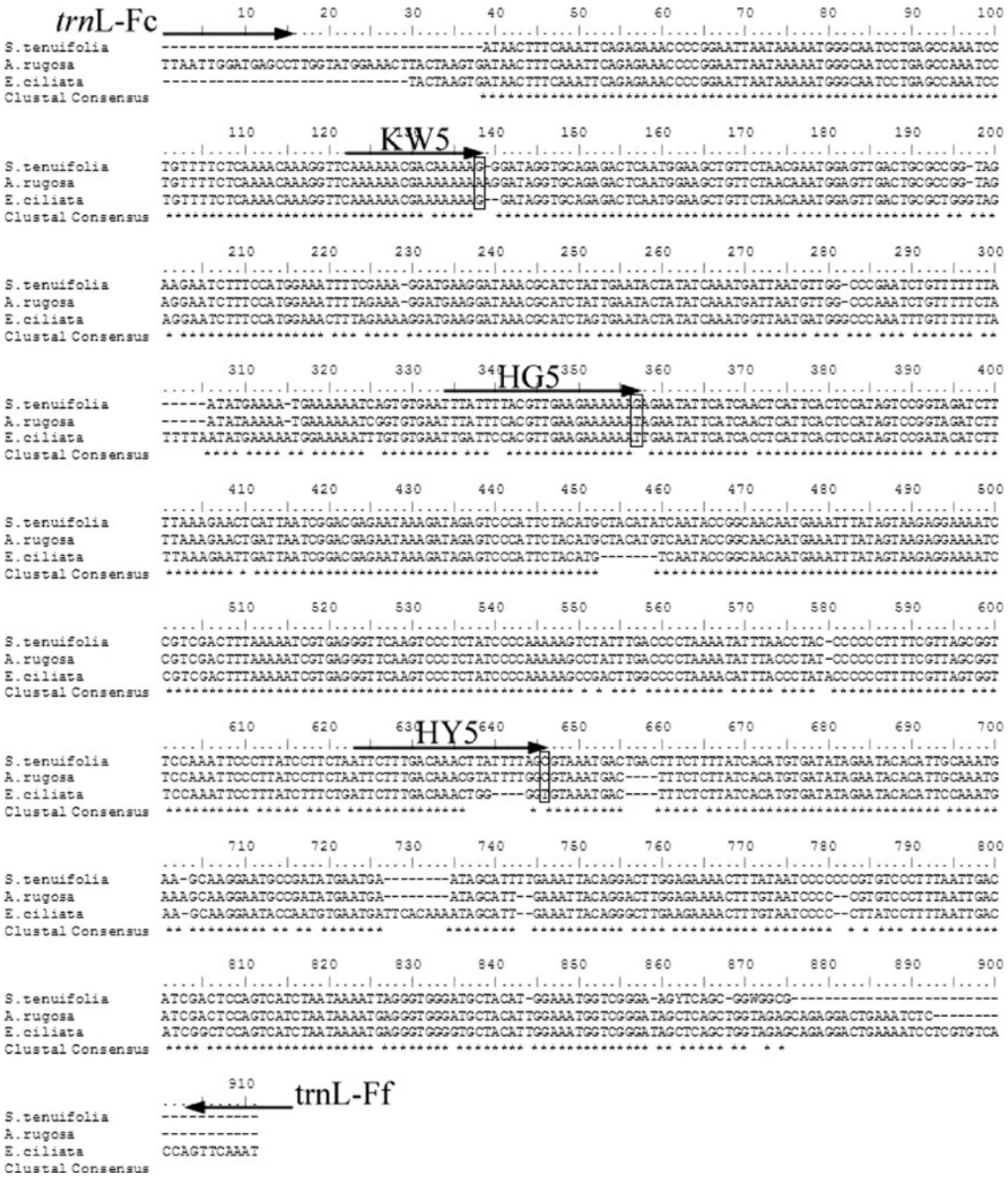

Fig. 2 DNA sequences in the *trn*L-F region for the *S. tenuifolia* ('Hyung-Gae'), *A. rugosa* ('Kwhak-Hyang') and *E. ciliate* ('Hyang-Yoo')

Results and discussion

Chloroplast DNA is much used for evolutionary and phylogenetic studies (Olmstead and Palmer 1994). The *trn*L-F region of the chloroplast DNA contains a *trn*L (UAA) intron and an intergenic spacer *trn*L (UAA)-*trn*F (GAA), which can easily be amplified using primers homologous to the exons of the *trn*L and *trn*F gene. This region has proved to be useful for the identification of many plants (Aagesen et al. 2005; Lang et al. 2006). Shaw et al. (2005) reported the relative utility of 21 non-coding chloroplast DNA sequences for phylogenetic analysis.

In this study, the *trn*L-F region was indeed useful for the identification of Hyung-Gae. The *trn*L-F regions of the 23 samples were sequenced. By using the universal primer sets of *trn*L-F region, '*trn*L-Fc' and '*trn*L-Ff', the amplified PCR products were around 900 bp. The *trn*L-F sequences of *S. tenuifolia* ('Hyung-Gae'), *A. rugosa* ('Kwhak-Hyang'),

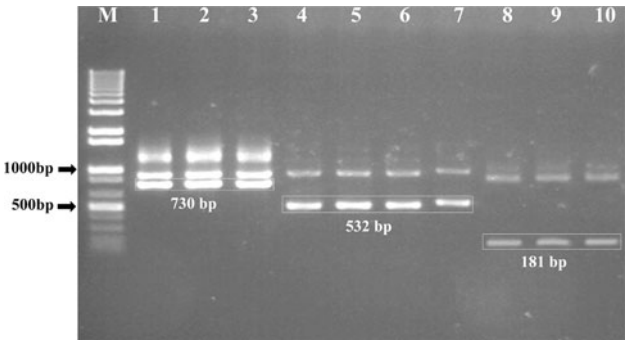

Fig. 3 The PCR products in multiplex PCR using the primers, '*trn*L-Fc', '*trn*L-Ff', 'HG5', 'KW5' and 'HY5'. *Lane M* 1-kb ladder, *lanes 1–3* various samples of *A. rugosa* ('Kwhak-Hyang') obtained from China and Korea; *lanes 4–7* four different samples of *S. tenuifolia* ('Hyung-Gae'); *lanes 8–10* various samples of *E. ciliata* ('Hyang-Yoo'). The 730-bp size band is specific for the detection of *A. rugosa* ('Kwhak-Hyang'), 532-bp size band is specific for the detection of *S. tenuifolia* ('Hyung-Gae'), and the 181-bp size band is specific for the detection of *E. ciliata* ('Hyang-Yoo')

and *E. ciliata* ('Hyang-Yoo') were deposited in GenBank with the accession numbers EU186386, EU244612, and FJ593412, respectively.

Multiple alignments of *S. tenuifolia* ('Hyung-Gae') and its adulterants were performed with the CLUSTAL X program. SNPs that were specific for each species were selected to design allele-specific primers. The specific primers, together with the universal primers of *trn*L-F region, were used to differentiate the three species, *S. tenuifolia*, *A. rugosa*, and *E. ciliate*, in multiplex PCR. The combination of these primers yielded expected amplicons with different sizes. As shown in Fig. 3, only samples of *S. tenuifolia* ('Hyung-Gae') generated a specific band of 532 bp. Likewise, samples of *A. rugosa* ('Kwhak-Hyang') and *E. ciliata* ('Hyang-Yoo') generated specific bands of 730 bp and 181 bp, respectively. Therefore, *S. tenuifolia* ('Hyung-Gae') and its adulterants can be clearly differentiated from each other by using their specific primers.

A host of molecular biological methods are now being used in identification of medicinal plants, including RAPD, RFLP, AFLP, etc., However, these methods are usually affected by DNA degradation, which often occurs in herbal products. Here, we have described a more reproducible and robust approach for the differentiation of *S. tenuifolia* ('Hyung-Gae') from *A. rugosa* ('Kwhak-Hyang') and *E. ciliate* ('Hyang-Yoo') by using SNP analysis. This method carries several advantages: first, they are sufficiently sensitive for identification with very small samples, and second, they can be applied to samples containing degraded DNA. Finally, DNA sequencing and comparison of the *trn*L-F regions with the sequences were stored in the GenBank database, enabling very reliable species identification. This method is highly accurate and time saving and can be used for numerous repeated tests of many medicinal commercial products. This is the first report of the molecular identification of *S. tenuifolia* ('Hyung-Gae'), and the whole scheme could be adapted for the development of new multiplex PCR systems for identification of other medicinal plant species.

Acknowledgments This study was supported by KGCMVP for Technology Development Program of Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea and was also supported by a grant (07092KFDA335) from the Korea Food and Drug Administration.

References

Aagesen L, Medan D, Kellermann J, Hilger HH (2005) Phylogeny of the tribe *Colletieae* (*Rhamnaceae*)—a sensitivity analysis of the plastid region *trn*L-*trn*F combined with morphology. Plant Syst Evol 250:197–214

Chang HM, But PPH (1986) Jingjie. Pharmacology and applications of Chinese materia medica. World Scientific, Singapore, pp 855–856

Chen SY, Li F (1993) A clinical guide to Chinese herbs and formulae. Churchill Livingstone, Edinburgh, UK

Choi YE, Ahn CH, Kim BB, Yoon ES (2008) Development of species specific AFLP-derived SCAR marker for authentication of *Panax japonicus* CA. MEYER. Biol Pharm Bull 31:135–138

Cui LCK, Yip KL, Dong TT, Tsim KW (2003) Authentication of *Panax notoginseng* by 5S-rRNA spacer domain and random amplified polymorphic DNA (RAPD) analysis. Planta Med 69:584–586

Fung D (2002) *Schizonepeta tenuifolia*: chemistry, pharmacology, and clinical applications. J Clin Pharmacol 42:30–36

Guo JX, Xie PS, Qi P, Wang ZT, Chen DF, Guo JI (1997) Herba Schizonepetae. Pharmacopoeia of the People's Republic of China. Chemical Industry, Beijing, China, pp 102–103

Ha WY, Shaw PC, Liu J, Yau FC, Wang J (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 TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hu J, Shi R, Zhang Y (2005) Content determination of luteolin and hesperidin in the effective fractions of spica *Schizonepeta* by HPLC. J Beijing Univ Tradit Chin Med 28:52–54

Hu J, Shi R, Zhang Y, Liu B, Dong Y (2006) Study on chemical compositions in the spikes of *Schizonepeta tenuifolia* Briq. J Beijing Univ Tradit Chin Med 1:38–40

Kirby AJ, Schmidt RJ (1997) The antioxidant activity of Chinese herbs for eczema and of placebo herbs-I. J Ethnopharm 56:103–108

Lang P, Dand F, Kubisiak TL (2006) Phylogeny of *Castanea* (*Fagaceae*) based on chloroplast *trn*T-L-F sequence data. Tree Genet Genomes 2:132–139

Lu JF, Zhang L, Feng YL, Ding AW, Du P (2002) Impact of lactones of *Schizonepeta tenuifolia* Briq. on plantar sweat gland and hemorrheology of rats. J China Pharm Univ 33:502–504

Matsuta M, Kanita R, Saito Y, Yamashita A (1996) The 3α-hydroxysteroid dehydrogenase inhibitory active flavonoids

and phenylpropanoids from *Schizonepeta* spikes. Nat Med 50:204–211

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

Olmstead R, Palmer JD (1994) Chloroplast DNA systematics: a review of methods and data analysis. Am J Bot 81:1205–1224

Oshima Y, Takata S, Hikino H (1989) Schizonodiol, schizonol, and schizonepetosides D and E, monoterpenoids of *Schizonepeta tenuifolia* spikes. Planta Med 55:179–180

Park IK, Kim LS, Choi IH, Lee YS, Shin SC (2006) Fumigant activity of plant essential oils and components from *Schizonepeta tenuifolia* against *Lucoriella ingenue* (*Diptera*: *Sciaridae*). J Econ Entomol 99:1717–1721

Sasaki Y, Komatsu K, Nagumo S (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 J, Lickey EB, Beck JT, Farmer SB, Liu W, Miller J, Siripun KC, Winder CT, Schilling EE, Small RL (2005) The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am J Bot 92:142–166

Shim YH, Choi JH, Park CD, Lim CJ, Cho JH, Kim HJ (2003) Molecular differentiation of *Panax* species by RAPD analysis. Arch Pharm Res 26:601–605

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol 17:1105–1109

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882

Wang J, Ha WY, Ngan FN, But PP, Shaw PC (2001) Application of sequence characterized amplified region (SCAR) analysis to authenticate *Panax* species and their adulterants. Planta Med 67:781–783

Yang F, Zhang R, Chen J, Yang C (2002) Monoterpenoids from *Schizonepeta tenuifolia*. Chin Tradit Herb Drugs 33:8–11

Yang J, Bao B, Zhang L, Ding A (2005) Quantitative analysis of pulegone in herba Schizonepetae. China J Chin Mat Med 30:669–670

Yen KY (1992) Schizonepetae herba seu flos, the illustrated Chinese materia medica—crude and prepared. SMC, Taiwan

Zhang Y, Zhou L, Shi R, Guo Y, Dong Y (2006) Study on chemical constituents in spikes of *Schizonepeta tenuifolia*. China J Chin Mat Med 15:1249–1253

Zheng HZ, Dong ZH, She J (1997) Jingjie. Modern study of traditional Chinese medicine. Beijing Xue Yuan, Beijing, China, pp 3072–3081

Zhu YP (1998) Jingjie. Chinese materia medica—chemistry, pharmacology, and applications. Harwood, Amsterdam, pp 60–61

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