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Molecular authentication of geo-authentic *Scrophularia ningpoensis*[*]

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Abstract: *Scrophularia ningpoensis* has long been used in the Chinese Materia Medica for inflammation. Like other herbal medicines, *S. ningpoensis* collected from different localities may considerably differ in their therapeutic efficacy, and the one grown in Zhejiang Province is recognized as geo-authentic. However, it is difficult to confirm the geographical authenticity by similar morphological characteristics. In the present study, inter-simple sequence repeat (ISSR) markers were conducted to detect *S. ningpoensis* from different origins. A 1259-bp fragment amplified by primer UBC874 was found only in geo-authentic ones. By cloning and sequencing that specific band, sequence characterized amplified region (SCAR) markers were designed to distinguish geo-authentic *S. ningpoensis* from others. This is a rapid and easy method that can be used to identify the geographical authenticity of *S. ningpoensis*.

Key words: Inter-simple sequence repeat (ISSR), Sequence characterized amplified region (SCAR), *Scrophularia ningpoensis*, Chinese Materia Medica, Traditional Chinese medicine

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1 Introduction

Scrophularia ningpoensis Hemsley, known as "Zhexuanshen", used in the Chinese Materia Medica (CMM), belonging to the family Scrophulariaceae, has a long history of widespread use in China (Kajimoto *et al*., 1989; Fernández *et al*., 1996; Miyase and Mimatsu, 1999; Giner *et al*., 2000). *S. ningpoensis* named by Forbes and Hemsley (1890) based on the specimens collected in Tiantong County, Ningbo City of Zhejiang Province, is endemic to China and now is widely cultivated in China as well. It is used to treat inflammation, laryngitis, tonsillitis, abscesses of carbuncles (Reid, 1996), and constipation (Yen, 1992). Recent research revealed that this medicinal species, which has high antiangiogenic activity, also can be used as an anticancer agent (Sagar *et al*., 2006).

The major bioactive components of *S. ningpoensis* have been reported to be harpagoside, angoroside C, acteoside, and cinnamic acid (Liu *et al*., 1995; Miyazawa *et al*., 1998; de Santos Galíndez *et al*., 2002; Díaz *et al*., 2004). However, determined by bioactive components, the quality and efficacy of CMM depend significantly on its geographical origin (Woo *et al*., 1999). The chemical differences of Radix Scrophulariae among various production regions were demonstrated to different extents. That grown in Zhejiang Province has better medicinal effect and is recognized as geo-authentic (Wang and Wang, 2007). Several methods based on high-performance liquid chromatography (HPLC) or combined with liquid chromatography-electrospray

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ionisation-mass spectrometry (LC-ESI-MS) were developed to quality and quantify the bioactive compounds in *S. ningpoensis* (Liu *et al*., 2007; Zhu *et al*., 2008). Our previous studies on HPLC fingerprints of *S. ningpoensis* have revealed that three of the four major bioactive compounds, harpagoside, angoroside C, and cinnamic acid, were largely variable among samples collected from different regions (Yang *et al*., 2010). The materials from Zhejiang Province produced the highest contents of the bioactive compounds, which have the most anti-inflammatory effect (Yang *et al*., 2010). Medicinal parts (roots) of *S. ningpoensis* originating from different geographical areas share similar morphological characters. Therefore, it is very difficult to distinguish *S. ningpoensis* of Zhejiang from others by using morphological methods.

The quality control of CMM is important for safe and effective use (Chung *et al*., 2006). Medicinal plants collected from different localities are considerably different in their therapeutic efficacy (Woo *et al*., 1999). Recent developments in molecular biology techniques make DNA markers be useful for the identification and standardization of CMM (Yang *et al*., 2001). Our group has established species-specific polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods for identifications of *Actinidia macrosperma* and *Sinopodophyllum hexandrum* (Gong *et al*., 2006; Zhao *et al*., 2007), and sequence characterized amplified region (SCAR) markers for *Sinocalycanthus chinensis* (Ye *et al*., 2006).

As to the identification of geo-authentic CMM, chemical fingerprints are the most used method, but the whole genome patterns are proven to be useful, accurate, and convenient as well. For instance, different arbitrarily primed (AP)-PCR fingerprints are used to distinguish samples of *Astragalus membranaceus* originated from different localities (Yip and Kwan, 2006). For *Codonopsis pilosula*, AP-PCR and random amplification of polymorphic DNA (RAPD) fingerprints revealed different patterns according to different geographic origins (Zhang *et al*., 1999). Similarly, *Vitex rotundifolia* samples from 14 different regions were divided by inter-simple sequence repeat (ISSR) markers (Hu *et al*., 2007). In this study, we used ISSR method to detect the whole genome of *S. ningpoensis* from different geographical origins and found a specific fragment only in populations originated from Zhejiang Province. Then based on that specific fragment, we designed a pair of diagnostic primers to identify *S. ningpoensis* of Zhejiang Province.

2 Materials and methods

2.1 Plant materials and DNA extraction

A total of 189 samples of *S. ningpoensis* originated from seven different Provinces were used in this study, wherein 85 individuals from three geographical origins were surveyed for ISSR and all samples were tested by designed SCAR primers (Table 1). The voucher specimens were deposited in the Herbarium of the Zhejiang University (HZU). DNA was isolated from silica-gel dried leaf by a modified hexadecyl trimethyl ammonium bromide (CTAB) method (Doyle, 1991).

Table 1 Sampling localities and codes of *S. ningpoensis*

Originated location	Locality code	Sample size	ISSR
Yaochuan, Pan'an County, Zhejiang	YC	15	√
Renchuan, Pan'an County, Zhejiang	RC	15	√
Guangmingcun, Pan'an County, Zhejiang	GM	15	√
Shanghu, Pan'an County, Zhejiang	PA	10	√
Xianju County, Zhejiang	XJ	10	√
Hubei	HB	10	√
Shanxi	SX	10	√
Jinfo Mountain, Chongqing	JF	19	
Jingang Mountain, Jiangxi	JX	15	
Pingjiang County, Hunan	HN	15	
Jiuhua Mountain, Anhui	AH	15	
Tianmu Mountain, Zhejiang	TM	15	
Dapan Mountain, Zhejiang	DP	10	
Matou County, Jiangxi	MT	15	

2.2 ISSR-PCR amplification

Out of 100 ISSR markers (UBC primer set No. 9, Biotechnology Laboratory, University of British Columbia, Vancouver, Canada; http://www.ubc.ca/), twelve primers (Table 2) that produced the strongest, clearest, and most reproducible bands were selected for further study. A 25 μl PCR amplification run

contained 25 ng of genomic DNA, 2.5 μl 10× buffer, 2 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 0.4 μmol/L of primers, and 2.0 U *Taq* DNA polymerase (Shanghai Sangon Biotechnology Co. Ltd., Shanghai, China). ISSR-PCR amplifications were performed in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Foster City, USA) with programme: 94 °C for 4 min; 45 cycles of 94 °C for 30 s, 49.4–65.0 °C for 45 s, and 72 °C for 1.5 min; 72 °C for 10 min (the specific annealing temperature for every ISSR primer is in Table 2). For every PCR run, a negative control without template DNA was also included. And every PCR amplification was repeated at least twice. PCR products were electrophoresed on 1.5% (v/v) agarose gels along with DNA Marker DL2000 (TaKaRa Biotechnology Co. Ltd., Dalian, China), then stained with ethidium bromide (EB), visualized with ultraviolet, and photographed.

Table 2 Twelve ISSR primers used in the present study to develop SCAR markers

Primer code	Sequence (5′–3′)	Annealing temp. (°C)
UBC809	AGAGAGAGAGAGAGAGG	60.5
UBC810	GAGAGAGAGAGAGAT	53.0
UBC811	GAGAGAGAGAGAGAC	52.7
UBC812	GAGAGAGAGAGAGAA	50.8
UBC827	ACACACACACACACACG	60.5
UBC834	AGAGAGAGAGAGAGAGYT	49.4
UBC855	ACACACACACACACACYT	62.0
UBC859	TGTGTGTGTGTGTGTGRC	57.0
UBC874	CCCTCCCTCCCTCCCT	65.0
UBC881	GGGTGGGGTGGGGTG	60.5
UBC887	DVDTCTCTCTCTCTCTC	50.8
UBC889	DBDACACACACACACAC	50.8

2.3 Cloning and sequencing of ISSR marker fragment

The specific band only amplified in *S. ningpoensis* of Zhejiang Province was excised from 2% (v/v) agarose gels and purified DNA fragment was cloned using pUCm-T vector (Sangon, Shanghai, China). The GENECLEAN II kit (BIO 101 Inc., Carlsbad, USA) is used to purify DNA fragment. Sequencing was run on an ABI 3700 Sequencer (ABU, Italy) by Shanghai Sangon Biotechnology Co., Ltd. Sequences were edited by SEQUENCHER (Version 4.0.5 Gene Codes Corporation, Ann Arbor, MI, USA).

2.4 Primer design and SCAR-PCR

Based on the sequencing results, a pair of primers (Table 3) was designed using the software of primer-primer 5.0 (Premier Biosoft International; Palo Alto, CA, USA) and synthesized by Shanghai Sangon Biotechnology Co., Ltd. The diagnostic PCR by primers CC874u and CC874d was carried out by programme: 94 °C for 5 min; 35 cycles of 94 °C for 30 s; 59 °C for 45 s; 72 °C for 1.5 min; 72 °C for 10 min. The reaction mixture is the same as ISSR-PCR, containing 0.2 μmol/L of the upper primer and 0.2 μmol/L of the lower primer. PCR products were run in 1.5% (v/v) agarose/EB gels.

Table 3 SCAR primers derived from cloned ISSR band of *S. ningpoensis* from Zhejiang Province*

SCAR primer	Sequence (5′–3′)
CC874u	CTATCATCGTCTTTGTCCATCC
CC874d	TGCTTTGAAACATTTGAACTTG

* ISSR primer: UBC874; Annealing temperature: 59.0 °C

3 Results

3.1 Screening the specific ISSR marker

In this study, 85 individuals originated from three different provinces (Table 1) were surveyed by ISSR-PCR to develop SCAR markers. ISSR-PCR results show that primer UBC874 provided an approximately 1300-bp band unique to populations originated from Zhejiang Province (Fig. 1).

3.2 Conversion of ISSR marker to SCAR marker

This specific band amplified by ISSR primer UBC874 was only found in the genome of *S. ningpoensis* from Zhejiang Province, but not in the genome of others. After gel-purified, cloned and sequenced, this DNA fragment turned out to be 1306 bp (GenBank accession No. EU082804.1; GI: 156185939). Based on analysis of that sequence, a pair of 22-bp SCAR primers (Table 3), CC874u and CC874d, was designed for the amplification of this DNA fragment. The upper primer was 70 bp from 5′ and the lower primer being 110 bp from 3′ amplified a 1126 bp fragment from samples of Zhejiang Province.

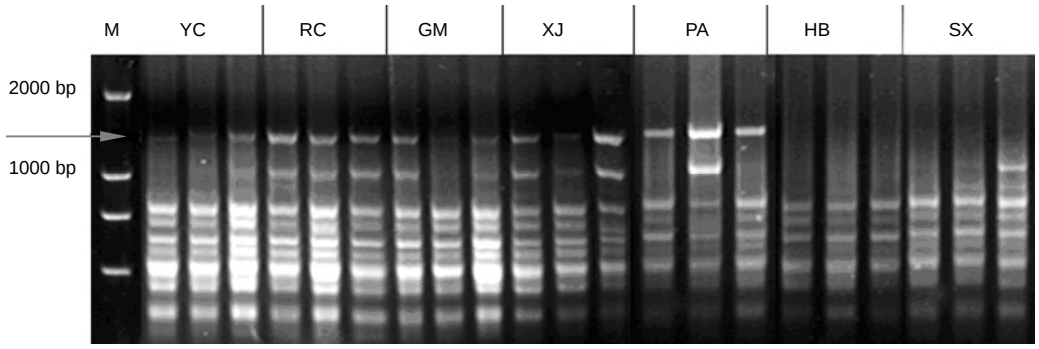

Fig. 1 PCR profiles of UBC874 in *S. ningpoensis*

Arrow indicates the specific band, which is only in samples from Zhejiang Province. M: DNA marker. YC, RC, GM, XJ, PA, HB, and SX are locality codes as shown in Table 1

3.3 Testing designed SCAR primers

All samples listed in Table 1 were amplified by SCAR primers CC874u and CC874d to test their specificities. PCR products showed that a single band about 1000 bp was only in accessions (Table 1) originated from Zhejiang Province (Fig. 2). Therefore, primers, CC874u and CC874d, designed in this study were proven to be diagnostic probe markers for identifying geo-authentic *S. ningpoensis* originated from Zhejiang Province.

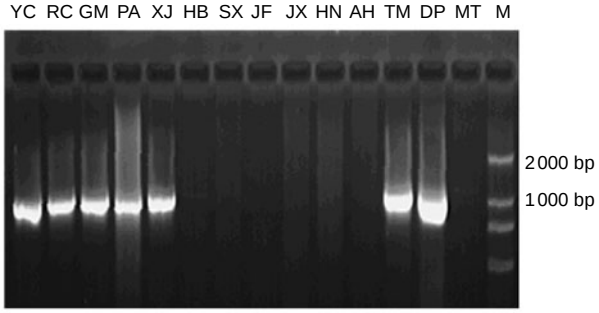

Fig. 2 Banding pattern of *S. ningpoensis* originated from Zhejiang Province (showing a distinct and reproducible band) and other provinces (showing no positive marker) with the designed primers CC874u/CC874d

M: DNA marker. YC, RC, GM, PA, XJ, HB, SX, JF, JX, HN, AH, TM, DP, and MT are locality codes as shown in Table 1

4 Discussion

Nowadays, with the booming of the herbal medicine market, standardization of traditional Chinese medicine has become more and more important. CMM is different from western and chemical medicines. The multiple sources and geo-authenticity of CMM generate unique confusion, e.g., different herbs species sharing one name, one herb using different names, and even one species collected from different localities having different medical effects (Zhao *et al*., 2006). *S. ningpoensis* has been widely used in CMM, but the geographical origin has always been a problem as mentioned above. Thus, to deal with the increasing dissatisfaction among consumers with the quality of herbal products and to obtain safe and effective application of CMM, an effective method to distinguish *S. ningpoensis* according to its geographical origins is critical (Moraes *et al*., 2005).

In general, morphological analysis, chemical chromatography, and DNA markers are used for authentication. Morphological method is conducted by observing, touching, smelling, and tasting (Zhao *et al*., 2006). Although, it is fast and easy, it also largely depends on personal experience. In many cases, morphological characteristics are often variable, and may disappear when crude drugs only contain the medical part like roots of a whole plant. And some herbs from the same genus or family are difficult to identify because of the similar morphological characteristics. In this study, all samples are the same species, *S. ningpoensis*, collected from different localities. Thus, it is almost impossible to distinguish geo-authentic *S. ningpoensis* from others by morphological method.

In recent years, chromatography has been widely used in the authentication of CMM (Hua *et al*., 2003). In particular, the HPLC chromatography fingerprinting technique, which can provide more precise information, is used for the identification of geographical origins within the same species (Lu *et al*.,

2005). HPLC is not perfect, however, and needs large amounts of samples. And the result is always influenced by harvest time, storage period, and processing method of CMM, since the chemical gradients of plants are easily affected by those factors (Lum *et al*., 2005). As to the process of HPLC experiments, standard chromatograms are first collected for a number of authentic species of samples. Then, four to nine characteristic peaks in a fingerprint chromatogram are chosen for authentication and identification purpose (Hu *et al*., 2005). Thus, this method is considered to be complicated and time-consuming.

In comparison to traditional and other existing methods, DNA markers have more advantages: sensitive, reliable, accurate, stable, convenient, and only a tiny amount of sample is sufficient. Among many types of DNA markers, for ISSR markers, no prior sequence reference is required, and they are more convenient than SSR and other markers (Zietkiewicz *et al*., 1994). Because of the longer primers and higher annealing temperature, ISSR can provide more reproducibility and stability than RAPD markers (Camacho and Liston, 2001). In addition, experiments of ISSR are much easier and less expensive than amplified fragment length polymorphism (AFLP) (Passinho-Soares *et al*., 2006). Considering the high reproducibility and polymorphic nature, the simple process of experiments, the stability, and low cost, using ISSR markers for CMM authentication is more practical and reasonable. A pair of primers developed in this study is an extension of ISSR markers. Although ISSR markers have the potential to provide a rapid, reliable, and simple authentication, as a universal primer, the specificity of ISSR is not as good as species-specific molecular markers. Also, in many cases, it can be an advantage to amplify a single fragment (Techen *et al*., 2006). This study provides a diagnostic PCR method to identify *S. ningpoensis* according to geographical origin by using species-specific molecular markers, and proves that DNA markers are much more useful for the identification of geographical origins than morphological and phytochemical methods.

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