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Identification of *Astragalus* Medicines Using Scar Markers

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ABSTRACT

Astragalus radix is a well-known herbal material in traditional Chinese medicine. *Astragalus membranaceous* and *A. membranaceous* var. *mongholicus* are known as the authentic origins of *Astragalus* medicines whereas *Hedysarum polybotrys* is a common adulterant of *Astragalus* radix in Taiwan. Random amplified polymorphic DNA (RAPD) primers were used to screen the polymorphic fragments. Among them, OPB-2 and OPC-14 directed the amplification of 3 specific fragments A1 (1.3 Kb), A2 (0.6 Kb) and H1 (0.8 Kb) from *A. membranaceous*, *A. membranaceous* var. *mongholicus* and *H. polybotrys* respectively, which were further converted to sequence characterized amplified region (SCAR) markers. Primer pairs HG3 and HG4 derived from A1, HG7 and HG8 from A2, and RC3 and RC4 from H3, were successfully employed on the authentication of *Astragalus* medicines and *H. polybotrys*.

Key words: *Astragalus*, authentication, polymorphism

INTRODUCTION

Astragalus Radix, also named as Huang Qi, is a well-known basic drug in traditional Chinese medicine. It has been formulated with other herbs and used as tonics for hundreds of years. In modern Chinese medicine, it is widely used as an immune-modulator or antioxidant in treating various chronic diseases such as cancer or cardiovascular diseases. The botanical origin of *Astragalus* Radix in Pharmacopoeia of China is the dried root of *Astragalus membranaceous* (Fisch.) Bge. and *A. membranaceous* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao[1]. However, there are many other species traded as *Astragalus* Radix; among them, *Hedysarum polybotrys* Hand.-Mazz. is the most common adulterant in Taiwan. Tung, *et al*.[2] reported that over 85% of *Astragalus* Radix sold in the local markets were *H. polybotrys*. In addition to minor anatomical differences, the *Astragalus* Radix contains stone cells but no calcium oxalate crystals in root[3] apart from *H. polybotrys*. Both *Astragalus* Radix and *Hedysarum* Radix own similar features, which make identification difficult.

The molecular markers in Chinese medicinal materials have been developed to identify many plant and animal species[4]. Polymorphic bands of *Astragali* radix identified by random amplified polymorphic DNA (RAPD) method were described by Cheng, *et al*.[5] and Na, *et al*.[6]. The disadvantage of this technique is poor fidelity since DNA quality plays a key role for the outcome of polymorphic bands[7]. The DNA extracted from dried herbal materials is usually damaged and contaminated with proteins, polysaccharides and secondary metabolites, which might affect the reliability of the RAPD results greatly. To improve the reproducibility of RAPD markers, the polymorphic bands generated from RAPD can be converted to the sequence characterized amplified regions (SCAR)[8], which is more specific than RAPD. It is reported that SCARs have several advantages over RAPD markers: (1) by using longer and sequence-specific PCR primers, SCARs improve the reproducibility of band polymorphism; (2) as the annealing temperatures are more stringent for SCARs than for RAPDs, only specific locus was detected by using each set of SCAR primers[9]. SCAR markers have been successfully developed to identify several Chinese medicinal materials, including *Panax* species[10], *Crocodilian* species[11] and snakes[12]. In this study, we demonstrated that three species-specific RAPD bands obtained by cloning and sequencing were converted to SCAR markers of *A. membranaceous*, *A. membranaceous* var. *mongholicus* and *H. polybotrys* for their authentication.

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MATERIALS AND METHODS

I. *Plant Materials*

Dried roots of *Astragalus membranaceous* (Fisch.) Bge., *A. membranaceous* (Fisch.) Bge. var. *mongholicus* Hsiao and *Hedysarum polybotrys* Hand-Mazz. were collected from Ji-Lin, Gan-Su and Shan-Xi provinces, China, respectively. Two sliced-root products of

Astragalus radix for fidelity test of SCAR primers were purchased from local stores. All samples were identified according to their anatomical characteristics[1,3]. Voucher specimens were deposited in the Development Center for Biotechnology, Taipei, Taiwan. Seeds of *A. spruneri*, *A. obseuru*, and *A. garbancillo* were gifts from the Millennium Seed Bank Project of the Kew Garden, UK. All seeds were surface sterilized and aseptically germinated on semi-solid MS medium[13] as described by Smith[14]. Three-month old micro-propagated plantlets were frozen with liquid nitrogen for further DNA extraction.

II. *DNA Extraction*

Dried roots of all samples were washed in tap-water and the outer skin was removed to avoid surface contamination. After being air dried, the samples were frozen with liquid nitrogen and ground into powder. Genomic DNA was extracted from the sample using DNeasy Plant Mini Kit (QIAGEN, Germany) by following the manufacturer's instructions. The DNA pellet was rinsed with 70% ethanol and resuspended in 50 μL TE buffer and stored at -20°C.

III. *RAPD Analysis*

One hundred RAPD primers (Operon Technologies, CA, USA) were initially screened to detect species-specific markers. The PCR reaction mix (50 μL) consisted of 100 ng of genomic DNA, 150 ng of RAPD primer, 1X PCR buffer, 1.5 mM $MgCl_2$, 250 μM of each dNTPs and 2.5 U of *Taq* polymerase (Bertec, Taiwan). Reaction took place in a TaKaRa TP600 thermal cycler (Japan) by the following program: 5 min at 95°C, followed by 30 amplification cycles of 95°C for 30 sec, 48°C for 30 sec, and 72°C for 1 min, and finally 10 min at 72°C for elongation. The PCR products were resolved by electrophoresis on 1.5% agarose (Sigma, USA) gel and in TAE buffer.

IV. *Cloning and Sequence of RAPD Products*

Three specific RAPD markers: A1 derived from the primer OPB-2 (5'-TGATCCCTGG-3') for *A. membranaceous* var. *mongholicus*; A2 and H1 derived from the primer OPC-14 (5'-TGCGTGCTTG-3') for *A. membranaceous* and *H. polybotrys*, were excised from the agarose gel and the DNA was purified using QIAquick Gel Extraction Kit (QIAGEN, Germany). The eluted fragments were ligated into pGEM T Easy Vector (Promega, USA), transformed into *Escherichia coli* strain DH5α and sequenced by ABI 3730 automated DNA sequencer (Applied Biosystems, USA).

V. *SCAR Analysis*

SCAR was amplified in a 50 μL PCR reaction mix consisting of 100 ng of genomic DNA, 150 ng of RAPD primers, 1X PCR buffer, 1.5 mM $MgCl_2$, 250 μM of each dNTPs and 2.5 U of *Taq* polymerase (Bertec, Taiwan). The cycling profile for *A. membranaceous* was: 5 min at 95°C, followed by 25 amplification cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, and finally 7 min at 72°C for elongation. The cycling profile for *A. membranaceous* var. *mongholicus* and *H. polybotrys* was: 5 min at 95°C, followed by 25 amplification cycles of 95°C for 30 sec, 64°C for 30 sec, and 72°C for 50 sec, and finally 10 min at 72°C for elongation.

RESULTS AND DISCUSSION

I. *Identification of Specific RAPD Markers*

RAPD method was performed in search for DNA polymorphic markers. Polymorphic profiles of *A. membranaceous* var. *mongholicus*, *A. membranaceous* and *H. polybotrys* were screened from the 100 RAPD primers (OPA1-20, OPB1-20, OPC1-20, OPD1-20, and OPE1-20). To increase the stringency between primers and genomic DNA, the annealing temperature was increased to 48°C which was higher than the normal condition and less polymorphic bands could be generated.

Table 1. Primer sequences used in the RAPD and SCAR analyses

Primer	Sequence
OPB-2	5'-TGATCCCTGG-3'
HG3	5'-CTATTAAAGACACATTCGGG-3'
HG4	5'-AACCGCAACGTCGAATCACC-3
OPC-14	5'-TGCGTGCTTG-3'
HG7	5'-ACTCGCGCAAGTTGGCTCCG-3'
HG8	5'-GTTGTTATGCTCCAGCGGGT-3'
RC3	5'-GTGCTTGGGCCAACATGGCT-3'
RC4	5'-GGGATGTCCGGGGAGAGTTA-3'

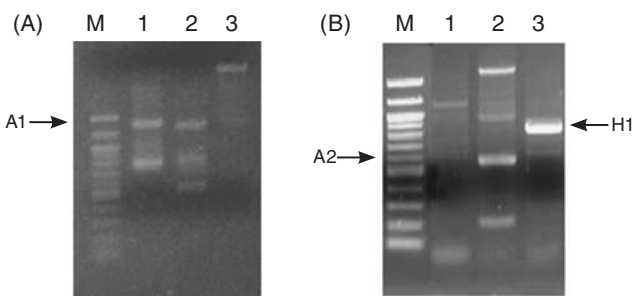

Figure 1. RAPD analysis of (A) OPB-2 primer; (B) OPC-14 primer. The arrows indicate the species-specific bands A1 (1.3 Kb), A2 (0.6 Kb) and H1 (0.8 Kb). Lane 1: *A. membranaceous* var. *mongholicus*; Lane 2: *A. membranaceous*; Lane 3: *H. polybotrys*; M: 100 bp molecular weight marker.

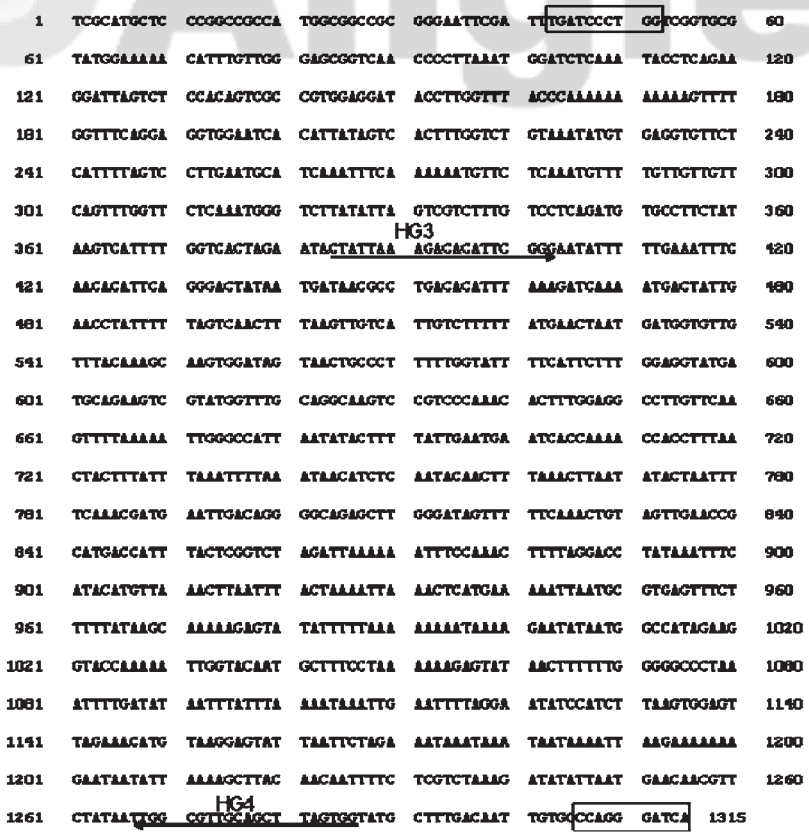

Figure 2. Sequence of *A. membranaceous* var. *mongholicus* fragment A1. SCAR was generated using primers HG3 and HG4 (underlined). RAPD primer OPB-2 is boxed.

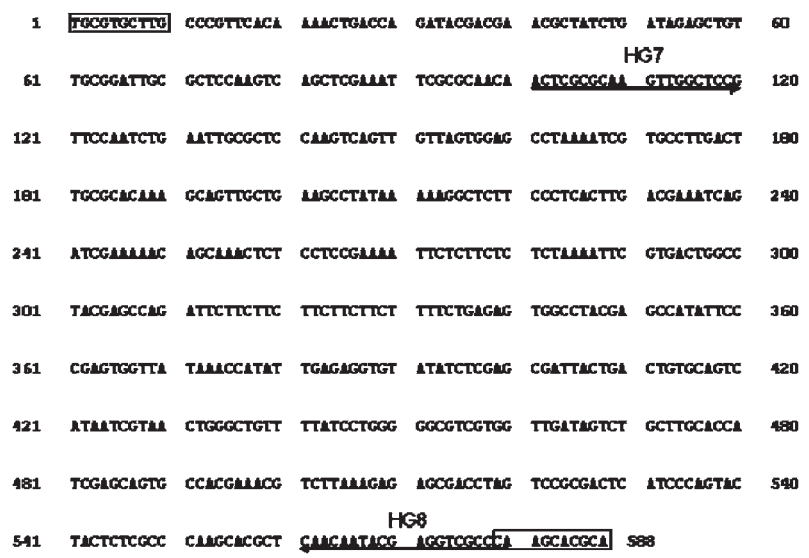

Figure 3. Sequence of *A. membranaceous* fragment A2. SCAR was generated using primers HG7 and HG8 (underlined). RAPD primer OPC-14 is boxed.

Most of the amplified fragments were within the range of 0.1 to 2 Kb. A number of putative species-specific bands were noted; however, most of them were faint or close to other bands. Only the fragments verified by repeated tests were considered to be potential markers. Using the RAPD primers OPB-2 and OPC-14 (Table 1), stable specific bands were identified (Figure 1). A 1.3 Kb polymorphic fragment A1 amplified by OPB-2 was specific to *A. membranaceous* var. *mongholicus* (Figure 1A, lane 1). A band amplified from *A. membranaceous* with similar size but slightly smaller than A1 was observed on lane 2. However, the band did not share any sequence similarity with A1 (data not shown). RAPD markers were not necessarily locus-specific[14]; polymorphic profiles could not be interpreted in terms of loci and alleles. In this case, fragments of similar size from lane 1 and 2 were not homologous. PCR of OPC-14 resulted in two specific bands, a 0.6 Kb fragment A2 was distinct to *A. membra-*

naceous, and a 0.8 Kb fragment H1 was to *H. polybotrys* (Figure 1B). These DNA fragments were subsequently cloned to pGEM T Easy Vector and subjected to sequence analysis.

II. *Sequencing and Amplification of SCAR Markers*

The sequences of RAPD fragments A1, A2 and H1, represented as specific markers of *A. membranaceous* var. *mongholicus*, *A. membranaceous* and *H. polybotrys*, were illustrated in Figures 2, 3 and 4, respectively. No significant sequence homology was identified among these sequences and the entries in GenBank. Primers of HG3 and HG4 from A1, HG7 and HG8 from A2, as well as RC3 and RC4 from H1 (Table 1) were designed and used to amplify these DNA fragments under high stringent conditions. Single bands of 0.9 Kb, 0.5 Kb and 0.7 Kb (Figure 5A, B and C) were obtained in *A. membranaceous* var. *mongholicus*, *A. membranaceous* and *H. polybotrys*, respectively as SCAR markers which were shorter than the original RAPD fragments A1, A2 and H1. Two SCAR analyses were performed to test the fidel-

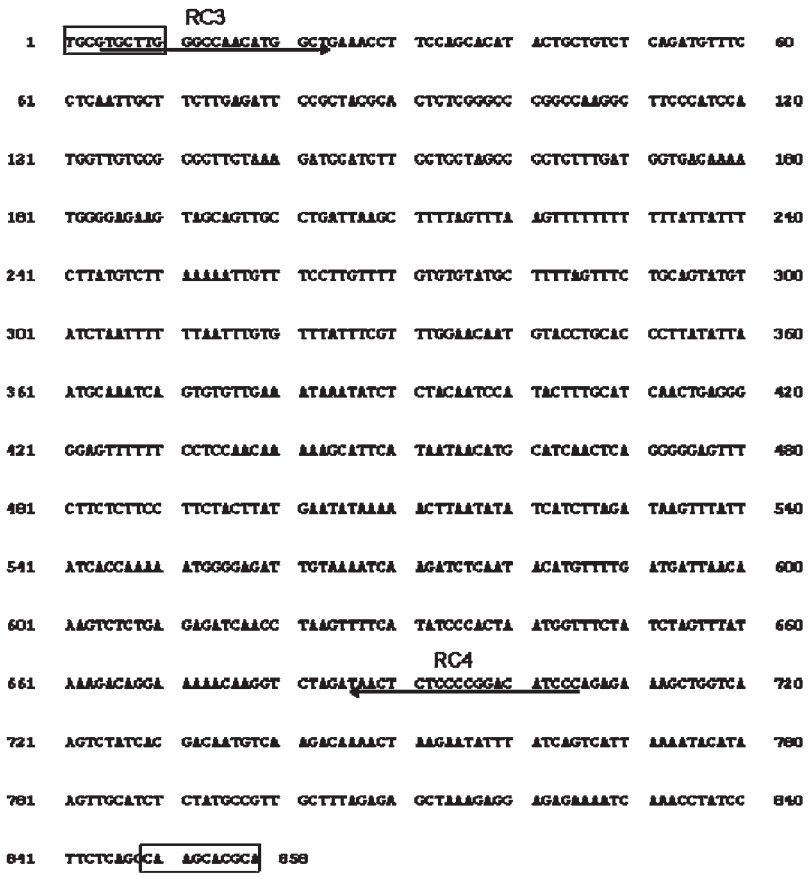

Figure 4. Sequence of *H. polybotrys* fragment H1. SCAR was generated using primers RC3 and RC4 (underlined). RAPD primer OPC-14 is boxed.

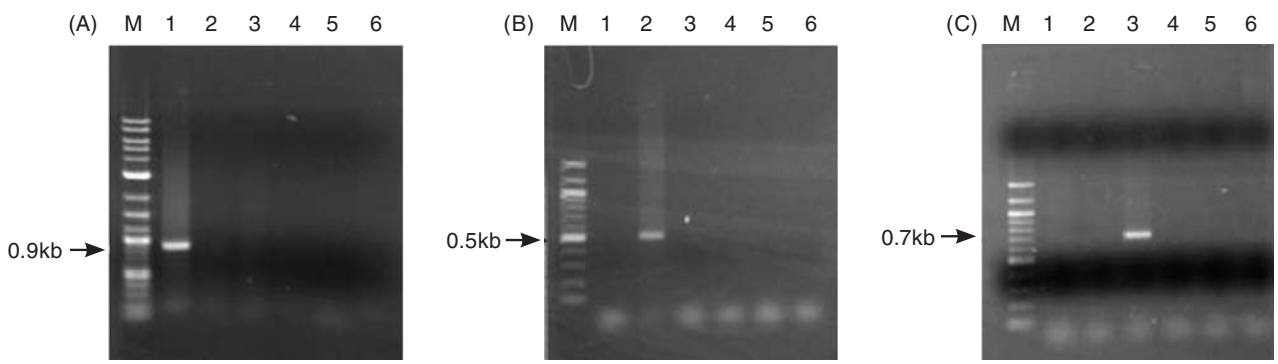

Figure 5. SCAR analysis of three other *Astragalus* plants using primers: (A) HG3 and HG4; (B) HG7 and HG8; (C) RC3 and RC4. Lane 1: *A. membranaceous* var. *mongholicus*; Lane 2: *A. membranaceous*; Lane 3: *H. polybotrys*; Lane 4: *A. spruneri*; Lane 5: *A. obseurus*; Lane 6: *A. garbancillo*; M: (A) 2-Log molecular weight marker; (B) and (C) 100-bp molecular weight marker.

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ity of SCAR markers. Firstly, DNA samples of three other *Astragalus* species *A. spruneri*, *A. obseuru*, and *A. garbancillo* extracted from fresh plants were examined by PCR. No band was found in these three *Astragalus* plants (Figure 5). SCAR markers were amplified only in the corresponding species rather than in other *Astragalus* plants. Secondly, two samples of *Astragalus* radix products purchased from local market were put to test. SCAR analysis indicated that one was *A. membranaceous* var. *mongholicus* (Figure 6, Lane 4) and the other was *H. polybotrys* (Figure 6, Lane 5). Under microscopic examination, one sample with grayish-yellow surface color and a brown peripheral cambium ring, containing stone cells but no calcium oxalate crystals was identified as *A. membranaceous* var. *mongholicus*. The surface color of the other sample was reddish-brown with calcium oxalate crystals fibers but no stone cells, was authenticated as *H. polybotrys*[1,3]. The microscopic examination of these two *Astragalus* radix samples agreed with the SCAR results.

Paran and Michelmore (1993)[8] indicated an advantage over SCARs by conversing a dominant RAPD marker into a codominant SCAR marker. However, in our study, the polymorphisms were retained as the presence or absence of bands when the corresponding SCAR primers were applied. Hence, these SCAR markers of *Astragalus* medicines would still be considered as dominant markers.

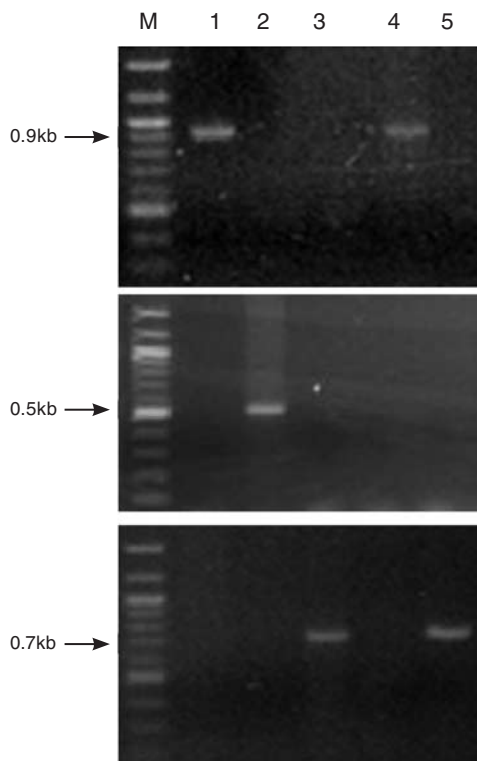

Figure 6. SCAR analysis for two Huang Qi samples using primers: (A) HG3 and HG4; (B) HG7 and HG8; (C) RC3 and RC4. Lane 1: *A. membranaceous* var. *mongholicus*; Lane 2: *A. membranaceous*; Lane 3: *H. polybotrys*; Lane 4 and 5: samples from local market. M: 100 bp molecular weight marker.

Several approaches were employed to study the phylogenetic relationships of *A. membranaceous* var. *mongholicus*, *A. membranaceous*, and *H. polybotrys*. The DNA sequences of 5S rRNA spacer, ITS, and 18S rRNA of these plant species and other *Astragalus* taxa were examined by Ma *et al.*[15] and Dong *et al.*[16]. Since *A. membranaceous* var. *mongholicus* was considered as a variety of *A. membranaceous*, they both share the greatest homology among those DNA sequences; rRNA sequences thus are not suitable to distinguish these two *Astragalus* medicines. It is likely that the rRNA sequences are more useful in identifying the inter-species rather than intra-species of *Astragalus spp.*

In conclusion, the identification and control of medicinal materials are closely connected to the efficacy of chinese medicine, which is the fundamental issue in the development of herbal drugs. Authentication of herbal material demands fast, reliable and reproducible protocols. In this study, we have demonstrated that two botanical origins of *Astragalus* radix and a common adulterant *H. polybotrys* could be distinguished by using sequence-specific designed SCAR markers.

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