Journal of Medicinal Plants Research Vol. 5(9), pp. 1677-1684 4 May, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Development of species and region specific random amplification of polymorphic DNA- sequence characterized amplified region (RAPD-SCAR) markers for identification of the genuineness of *Spica prunellae* (Lamiaceae)

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Accepted 23 February, 2011

The present study was carried out to develop appropriate molecular markers to distinguish genuine herbal medicine *Prunella asiatica* growing in Jiangsu province at both species and population level. Two putative markers, which are specific for *P. asiatica* species and its habitat respectively, were identified by random amplification of polymorphic DNA (RAPD). Respectively, two sequence characterized amplified region (SCAR) markers were further developed from the two RAPD markers above. The amplification using *P. asiatica*-specific SCAR marker showed that *Prunella vulgaris, P. asiatica* and *Prunella hispida* could be regarded as one species in the taxonomy. The amplification of another SCAR marker was closely linked to region-specific. The results showed that this newly developed molecular marker method could be considered as a convenient and reliable method for the genuineness identification *of Spica prunellae*. Moreover, the taxonomic modification of *Prunella* species including *P. vulgaris, P. asiatica* and *P. hispida* was also discussed in this paper.

Key words: *Prunella asiatica*, species-specific, region-specific, random amplification of polymorphic DNA - sequence characterized amplified region marker.

INTRODUCTION

Prunella, which is included in the Chinese Pharmacopoeia as having the action of moistening the lung and resolving phlegm, subduing hyperactivity of the liver and counteracting toxicity, tonifying kidney and harmonizing stomach, is one of the most important traditional Chinese medicines with respect to both its distribution and medical importance. The officinal part of these plants is the dry ripe *Spica* of *Prunella vulgaris* Linn. (Lamiaceae) (The Pharmacopoeia Committee of PRC, 2005). The main distribution region of this species is located in Jiangsu, Anhui, Hubei and Henan provinces in China (Xiao, 2002). *Prunella* also contains *Prunella asiatica* Nakai, *Prunella hispida* Benth. and a cultivar *Prunella grandiflora* (Linn.) Jacq. (Wu and Li, 1977). Recently, based on both molecular (ITS) data and detailed morphological comparison, *P. asiatica* could be regarded as one species as *P. vulgaris*, while *P. hispida* could be treated as a variety of *P. vulgaris* (Wei et al., 2008).

Nanjing area in Jiangsu province is well known as ideal region for growing of genuine herbal medicine Xiakucao (also named as Jingxiakucao), and the actual *Prunella* species here has been identified as *P. asiatica*. Due to their similar morphology, the dry ripe spicas of almost all *Prunella* species (*P. asiatica*, *P. vulgaris* and *P. hispida*), and even two species of another genus of same family – *Ajuga decumbens* Thunb. and *Ajuga multiflora* Bunge

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Table 1. Taxon and sampling sites of plants.

Taxon	Origin	Voucher number
P. asiatica Nakai	Jurong, Jiangsu Province	20050901-20050907
	Pan'an, Zhejiang Province	20051001-20051007
	Wuhu, Anhui Province	20051008-20051014
	Xishui, Hubei Province	20051101-20051107
P. vulgaris Linn.	Xinyu, Jiangxi Province	20051108-20051114
	Hunan, Province	20051201-20051207
	Yingxiu, Sichuan Province	20060701-20060707
	Guiyang, Guizhou Province	20060301-20060307
P. hispida Benth.	Kunming, Yunnan Province	20060708-20060714
P. grandiflora (L.) Jacq.	Nanjing, Jiangsu Province	20051115-20051121
A. decumbens Thunb.	Nanjing, Jiangsu Province	20051122-20051128
A. multiflora Bunge	Jurong, Jiangsu	20060401-20060407

(Lamiaceae) are often mistakenly regarded as genuine Xiakucao herbal medicine in current market (Wang et al., 1994a, b, 1996a, b). Since the quality and therapeutic effect of herbal medicine are closely related to the genuineness of species and their ideal habitats, it is necessary to find a way to distinguish genuine herbal medicine species *P. asiatica* growing in Jiangsu area from others at the population level.

DNA polymorphisms and other molecular markers have been used from a long time for genetic study and identifications. Random amplified polymorphic DNA (RAPD) markers are based on amplification of polymorphic DNA. Molecular markers have been used for identification of genuine traditional Chinese medicines at both species and population level (Liu et al., 2001, 2006; Xu et al., 2001; Wang et al., 2003; Yu et al., 2003). Because most RAPD markers are dominant and thus do not distinguish between heterozygous and homozygous alleles, RAPD markers are often converted into sequence characterized amplified region (SCAR) markers. SCAR markers are obtained by amplification of genomic DNA using a pair of specific primers. For its stability and repeatability, SCAR has been adopted to identify genuineness of many species, such as *Panax* (Wang et al., 2001) and *Atractylodes* (Huh and Bang, 2006).

The objective of this research was to develop identification methods based on DNA fingerprints and specific molecular markers, in order to provide for the rapid and effective identification, classification and resource protection of *Prunnella* specices.

MATERIALS AND METHODS

The plants used in this study are listed in Table 1.Fresh leaves were collected from *P. asiatica*, *P.* vulgaris, *P. hispida*, *P. grandiflora*, *A. decumbens* and *A. multiflora* growing in different regions in China (Table 1). Plants were authenticated and deposited at Herbarium of Institute of Botany, Chinese Academy of Sciences, Jiangsu, China (CAS).

DNA isolation

DNA was extracted from fresh or silica gel-dried leaves by the modified CTAB (Cetyl Trimethyl Ammonium Bromide) method (Doyle and Doyle, 1987), and further purified by using a DNA purification Kit (Beijing Bio-lab Materials Institute, China). The purified DNA was then dissolved in sterile water and stored at -20°C until use. The concentration of the DNA was determined by 0.8% agarose gel stained with ethidium bromide.

RAPD analysis

The RAPD analysis was carried out using 120 primers. Reaction contents contained 1 x Mg-free DNA polymerase buffer, 2.5 mmol/L $MgCl_2$, 1 U *Taq* DNA-polymerase, 30 ng template DNA, 0.4 µmol/L each primer and 0.15 mmol/L dNTPs. The polymerase chain reaction (PCR) was conducted with the following program: a premelt of 3 min at 94°C, followed by 40 cycles of 45 s denaturation at 94°C, 30 s annealing at 38°C, 1 min 30 s extension at 72°C, plus a final extension of 5 min at 72°C. Each 20 µl all PCR products were separated on a 1.4% agarose gel, visualized with ethidium bromide staining under ultraviolet light.

SCAR analysis

The two amplifed fragments of RAPD markers were excised from the agarose gel and purified with the DNA purification system according to manufacturer's instructions (TAKARA, Dalian, China). The PCR products were directly sequenced (Bioasia, Shanghai, China). Respectively, two pairs of sequence-specific primers were devised from the the 5' and 3' milo ends of the two RAPD markers by using the primer 5.0. Primer F (5'-TCTCTAGGGGTAGCCCAGAAAC-3') and R (5'-TCTCTAGGGGCTTCTAGAATCTTC-3') were used for PCR amplification of genomic DNA from totally 80 *Prunella* and *Ajuga* individuals; F_2 (5'-CTTCTCGGTCTTTGTTTTTACAGA-3') and R_2

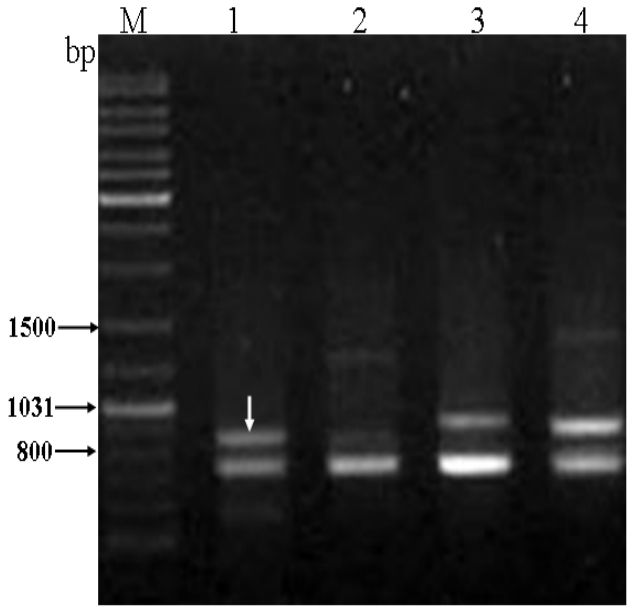


Figure 1. An RAPD assay for species-specific. DNA was amplified using the primer AU06. M. Marker; (1) *P. asiatica,* (2) *P. vulgaris*, (3) P. hispida, (4) *P. grandiflora*.

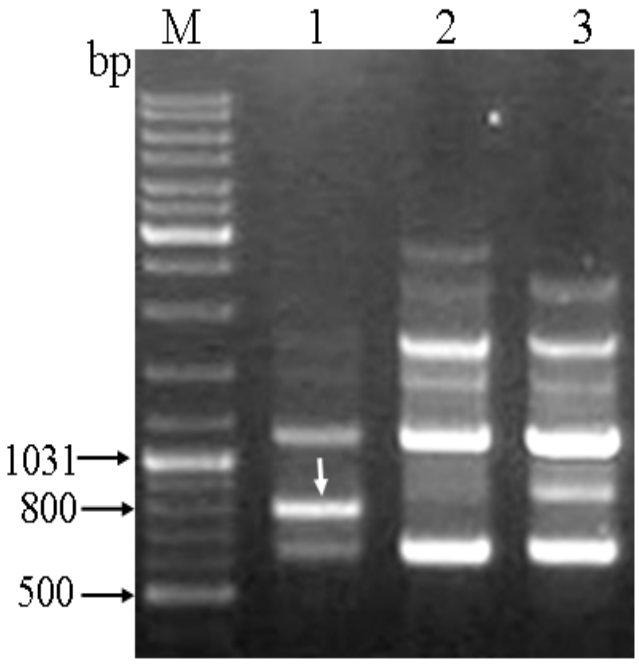


Figure 2. An RAPD assay for region-specific. DNA was amplified using the primer AU11.M. Marker, (1) Jiangsu, (2) Hubei, Zhejiang, Sichuan, Anhui and Hunan, (3) Yunnan.

(5'-CTTCTCGGTCCATCAAGTATTTG-3') were used for authentication of *Prunella* species (including 63 individuals) from different locations of China. PCR reactions were performed in a 20 µl reaction mixture containing 1 x Mg-free DNA polymerase buffer, 2.5 mmol/L $MgCl_2$, 1 U *Taq* DNA-polymerase, 60 ng template DNA, 0.3 µmol/L each primer and 0.15 mmol/L dNTPs. The parameters used were 94°C for 3 min; 35 cycles at 94°C for 45 s, 58°C for 30 s, and 72°C for 60 s, and final extension at 72°C for 5 min. PCR products were separated on a 1.4% agarose gel, visualized with ethidium bromide staining under ultraviolet light.

RESULTS

Identification of RAPD marker

DNA pools of 80 plants were screened with a total of 120 primers. Figure 1 showed the specific molecular marker that was indicated with arrows. A polymorphic fragment of 800～900 bp linked to species-specific was observed in *P. asiatica* but not in the rest *Prunella* species when using primer AU06 (5′-TCTCTAGGGG-3′). Therefore this specific RAPD marker can be used as molecular markers for species identification and resource protection of *P. asiatica*. Then DNA of *P. asiatica* individuals from 3 regions in China was respectively screened with a total of 120 primers. An 800 bp fragment was only amplified in sample from Jiangsu but not in the rest samples from other two regions when using primer AU11 (5′-CTTCTCGGTC-3′) (Figure 2). Therefore this amplified product was selected as putative region-specific RAPD marker for *P. asiatica*.

Conversion of RAPD marker into SCAR markers

Two RAPD markers were sequenced, their sizes were 860 and 792 bp (Figures 3 and 4), respectively. But no homology was found between the sequenced marker fragments and known sequences in the database using BLAST. Two pairs of SCAR-PCR primers were designed and synthesized according to the end-sequences of the two RAPD markers. The F/R primers contained the original RAPD primer sequences of AU06 at their 5′ ends, respectively, and therefore the fragment produced after amplification with the SCAR primers F/R was 860 bp, the same length as the original RAPD marker. Genomic DNAs of *Prunella* (6 samples) and *Ajuga* species (74 samples) were screened with primer F and R. As shown in Figure 5, a bright band of 860 bp was obtained in all the *P. asiatica* individuals from Jiangsu, Zhejiang and Anhui province except those from Hubei (7 individuals of each province). It was strange that this SCAR band was observed in all *P. hispida* individuals from Yunnan, as well as in all *P. vulgaris* individuals from Jiangxi and *P. vulgaris* individuals from Hunan (7 individuals of each province). As expected, the SCAR band was absent in *P. grandiflora*, *A. decumbens* and *A. multiflora*.

The F2/R2 primers contained the sequences of AU11 at their 5′ ends; therefore, the produced fragment after amplification using the primers F2/R2 was 792 bp (Figure 6), the same length as the original RAPD marker.

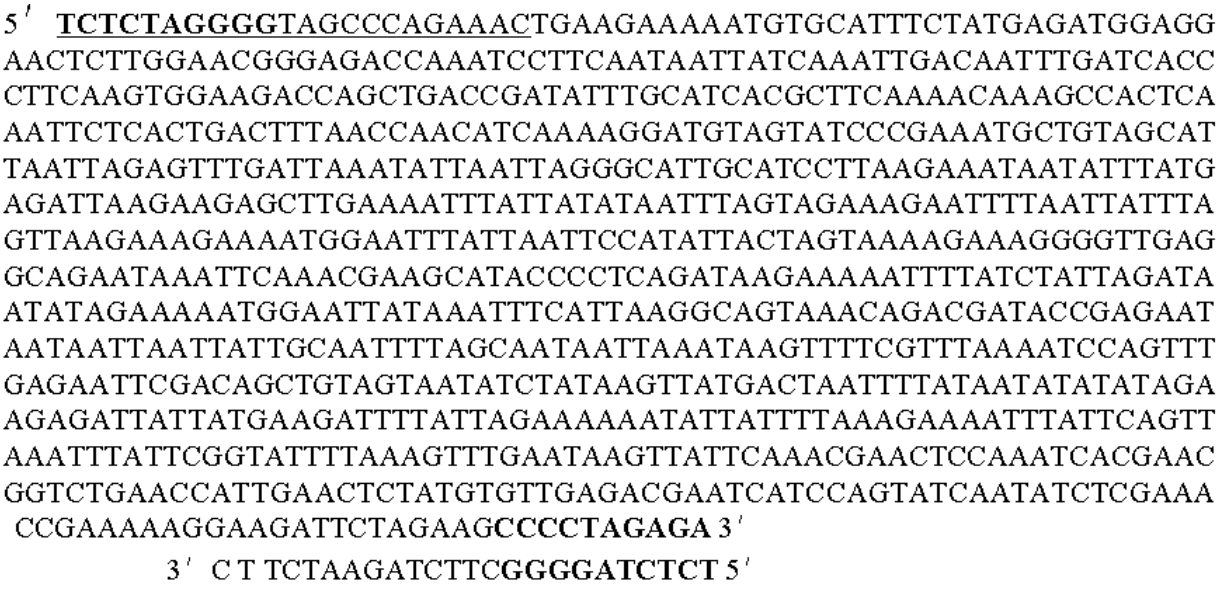


Figure 3. Nucleotide Sequence of RAPD marker amplified from *P. asiatica*. Nucleotides in brackets represent the partial complementary sequence of the 3′ end, those underlined show the RAPD primer AU06 and the nucleotides in bold indicate the designed SCAR marker.

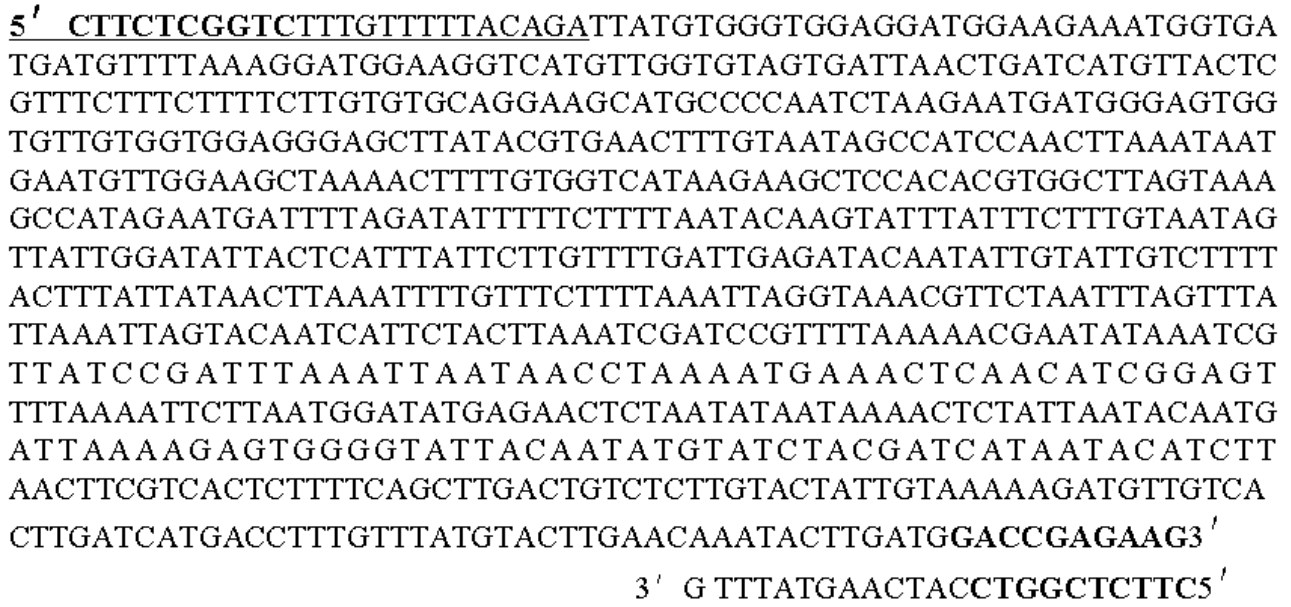
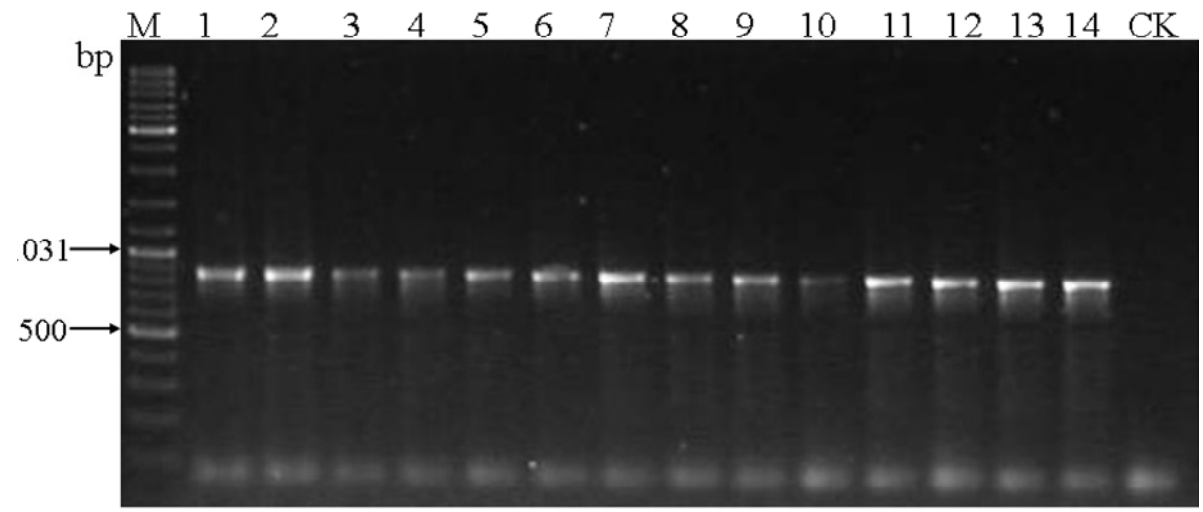


Figure 4. Nucleotide Sequence of RAPD Amplicon Specific to the *P. asiatica* from Jiangsu Province. Nucleotides in brackets represent the partial complementary sequence of the 3′ end, those underlined show the RAPD primer AU11 and the nucleotides in bold indicate the designed SCAR marker.

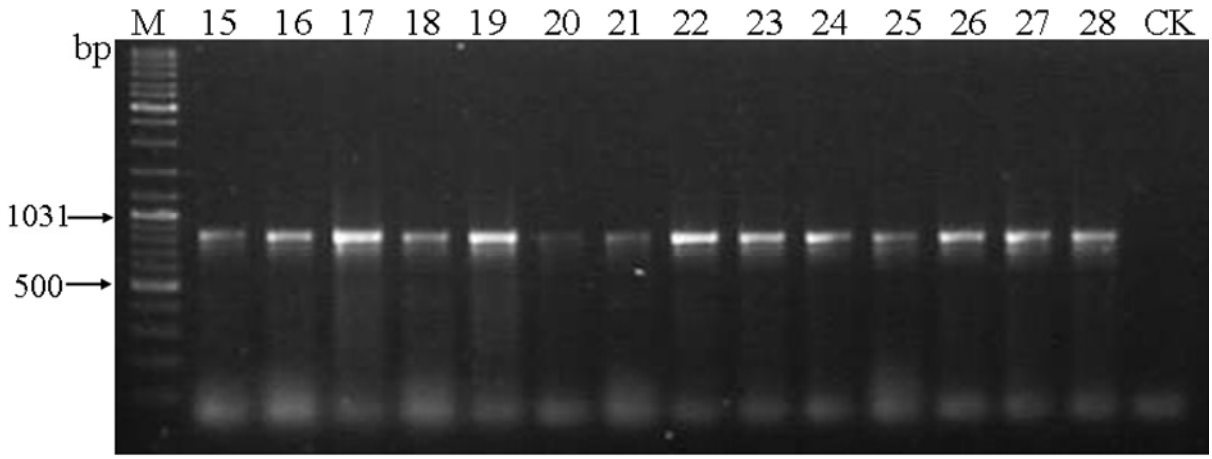
Genomic DNAs of *Prunella* (7 samples) growing in Jiangsu. 63 samples of *Prunell Linn.* from different regions were screened with primer F2 and R2. A bright band of 792 bp was only obtained in individuals from jiangsu but absent in the rest individuals from other regions.

DISCUSSION

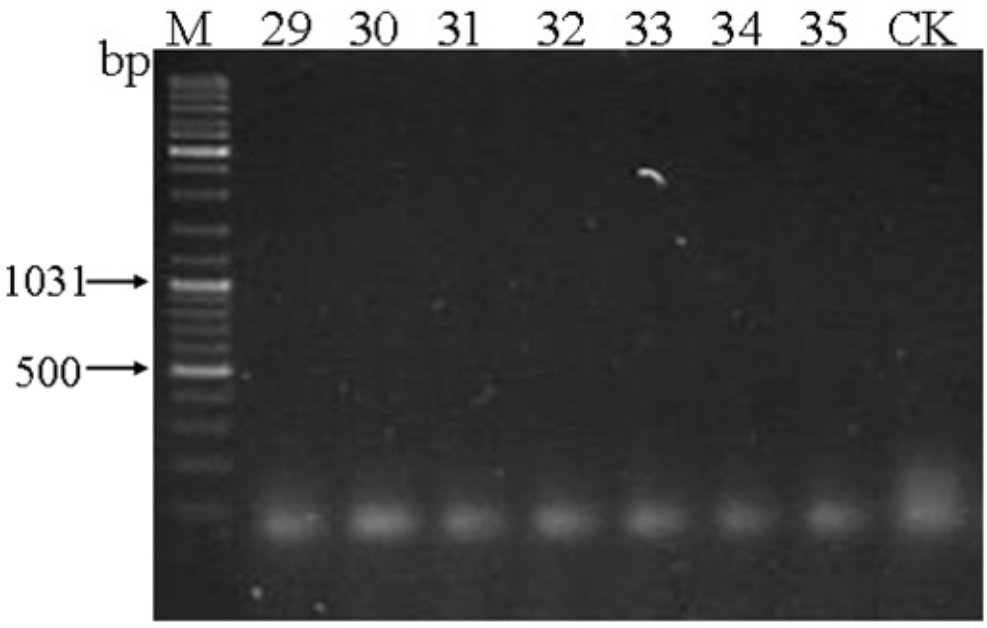
In our study, a molecular marker method for *P. asiatica* identification was developed based on RAPD-SCAR analysis, and a region-specific molecular marker was developed simultaneously. The newly developed


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 CK
bp
1031
500

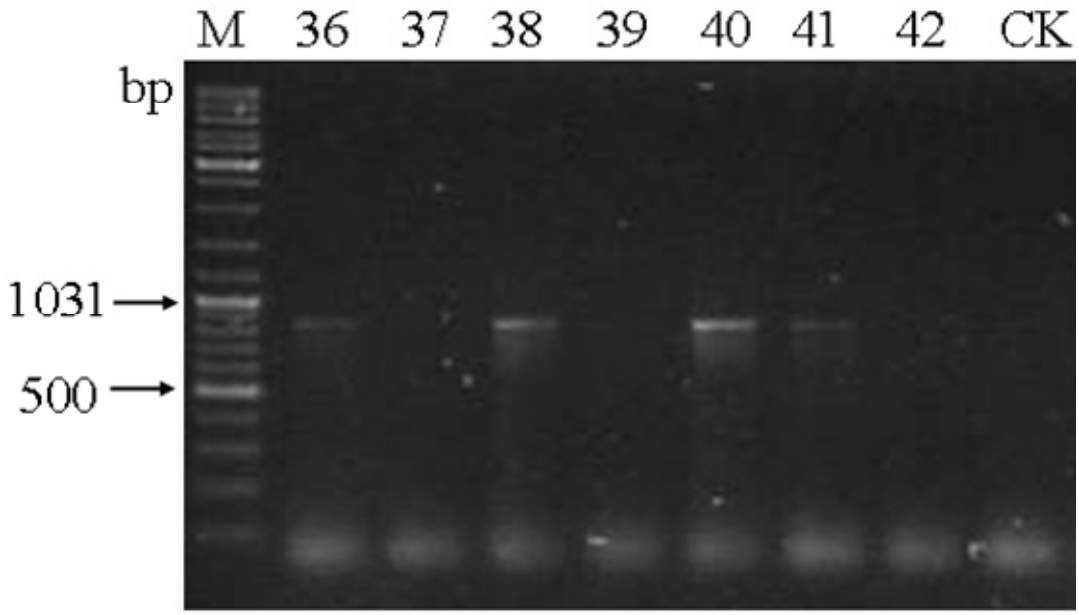
A


M 15 16 17 18 19 20 21 22 23 24 25 26 27 28 CK
bp
1031
500

B


M 29 30 31 32 33 34 35 CK
bp
1031
500

C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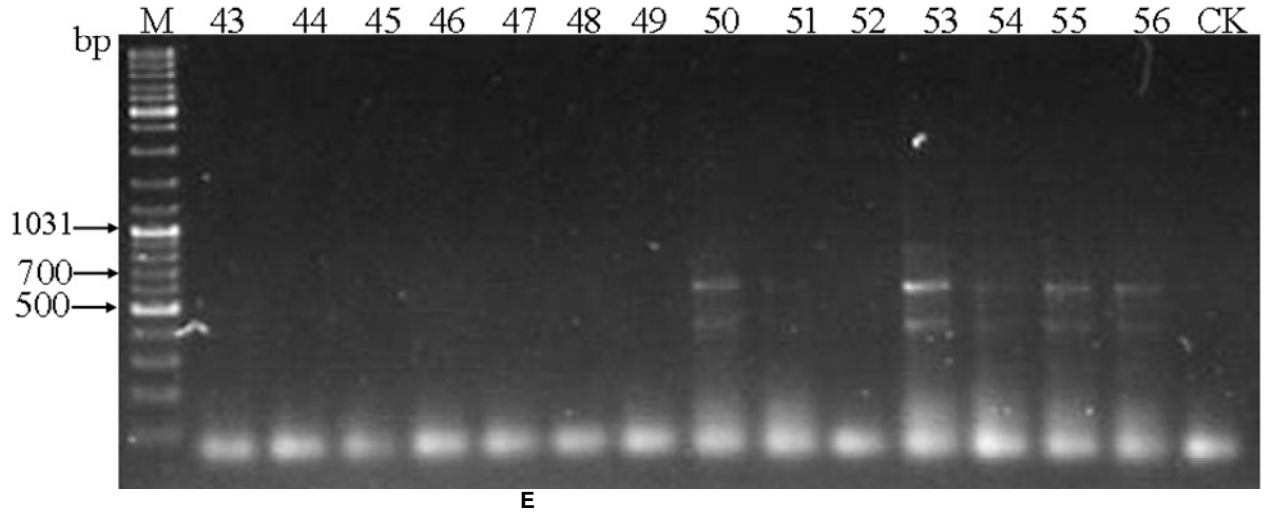
bp
1031
500

D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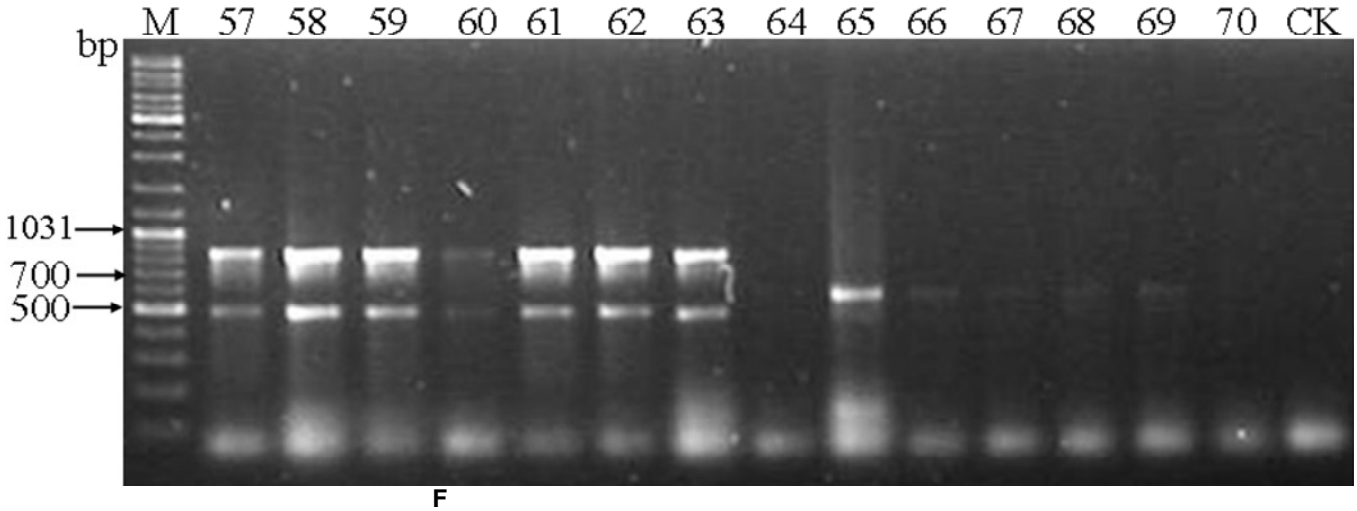
bp
1031
700
500

E


M 57 58 59 60 61 62 63 64 65 66 67 68 69 70 CK
bp
1031
700
500

F

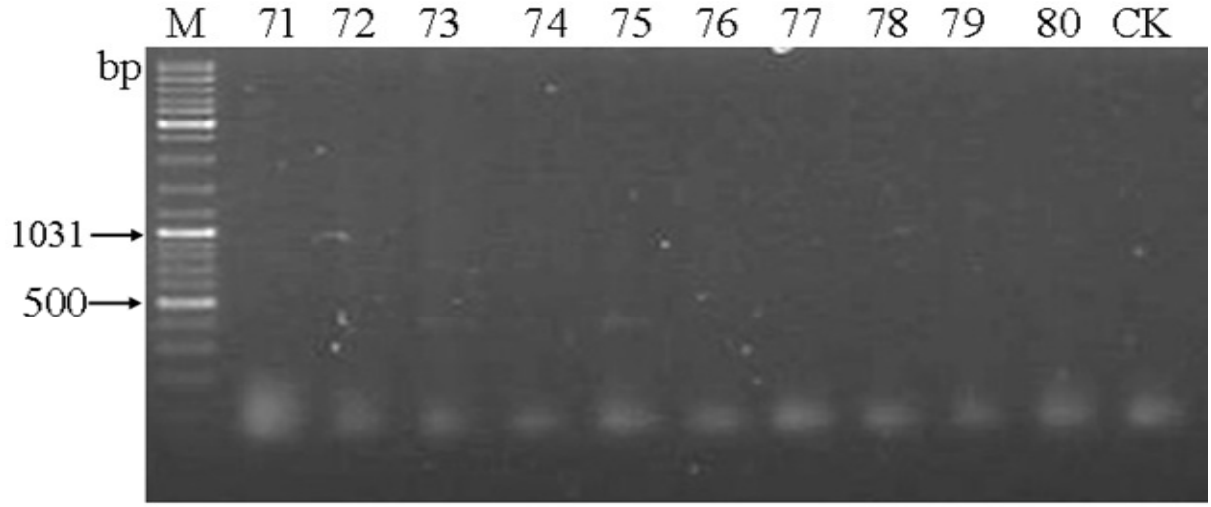


Figure 5(A-G). PCR Amplification of six *Prunella* and *Ajuga* species using F and R in 1.4% Agarose Gel. M. Marker; 1-7. *P. asiatica* from Jiangsu; 8-14. *P. asiatica* from Zhejiang; 15-21. *P. asiatica* from Anhui; 22-28. *P. vulgaris* from Jiangxi; 29-35.*P. asiatica* from Hubei; 36-42. *P. vulgaris* from Hunan; 43-49. *P. vulgaris* from Sichuan; 50-56. *P. vulgaris* from Guizhou; 57-63. *P. hispida* from Yunnan; 64-70. *P. grandiflora* from Jiangsu; 71-75. *A. decumbens* from Jiangsu; 76-80. *A. multiflora* from Jiangsu; CK. Negative control.

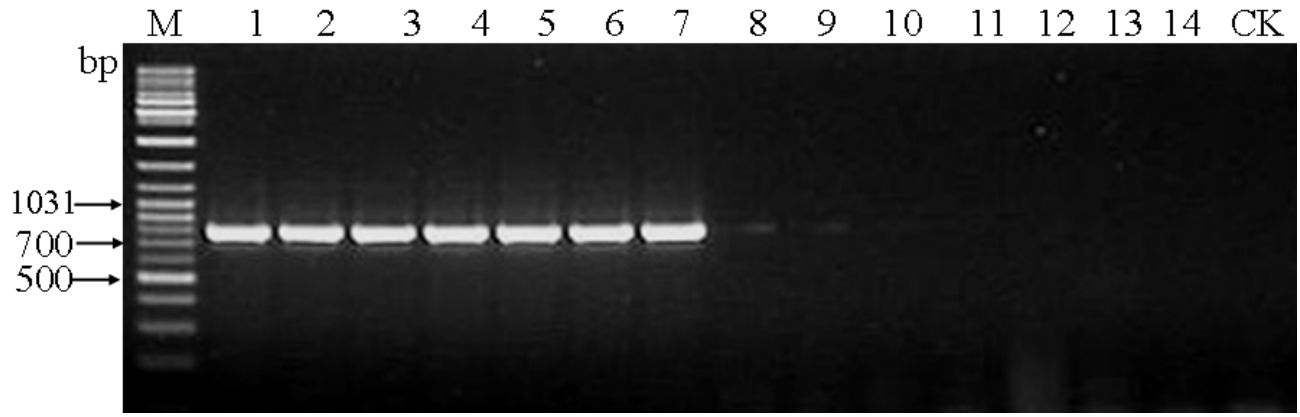


Figure 6. SCAR Analysis of region-specific using primer F_2 and R_2. M. Marker, 1-7. Jiangsu, 8-14. Zhejiang, CK. negative control.

molecular marker methods gave better and more reliable identification of *P. asiatica*. However, there were something unexpected occurred in the amplification of individuals using *P. asiatica*-specific SCAR primer. The SCAR band was absent in individuals of *P. asiatica* from Hubei but present in all *P. hispida* individuals from Yunnan, as well as in all the *P. vulgaris* individuals from Jiangxi and four *P. vulgaris* individuals from Hunan, unexpectedly. Refer to the taxonomic modification of *Prunella* species (Wei et al., 2008), that there is no distinct or essential difference among *P. vulgaris, P. asiatica* and *P. hispida*. Therefore, the RAPD-SCAR marker developed from amplicon using primer AU06 was not very effective to distinguish *P. asiatica* from the other two, as they are supported by molecular data to be the same or similar taxon (Wei et al., 2008). Therefore we suggest that *P. vulgaris, P. asiatica* and *P. hispida* could be all treated as general *P. vulgaris* for (*P. vulgaris was* named firstly).

Due to high similarity of spica morphology among *P. vulgaris, P. asiatica*, *P. hispida* and another two *Ajuga* species, it is difficult to distinguish genuine source in Jiangsu from the others. In fact, only the genuine source – *P. asiatica* growing in its ideal habitat (Jiangsu province) has best quality and therapeutic effect. In this study, the second SCAR marker was Jiangsu-specific and could be

used as an effective tool to identify genuine *P. asiatica* distributed in Jiangsu. Compared with RAPD markers,SCAR markers is more stable, repeatable and convenient in practical application. They can be directly used in practice.

Conclusion

The species- and region-specific RAPD-SCAR markers have been developed for identification of the genuineness of *S. prunellae* in *P. vulgaris* and related five species. The results indicated that the newly developed molecular marker methods could be considered as a convenient and reliable method of identification for the genuineness of *S. prunellae*. Moreover, the taxonomic modification of *prunella* species including *P. vulgaris, P. asiatica* and *P. hispida* was also discussed in this paper. The applicable prospects of these medicinal plants with genuine origin would be extensive and diverse in further studies.

ACKNOWLEDGEMENTS

Financial supports from the Natural Science Fund (Grant. NO. BK2005206) and the Fund for constructing resource library of genuine medicinal materials of Jiangsu (Grant. NO. BM2006104) of the Science and Technology Department of Jiangsu province, China are highly appreciated.

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