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Supplementary material can be obtained from the authors on request.

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## Development of SCAR marker for authentication of *Pueraria tuberosa* (Roxb. ex. Willd.) DC.

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***Pueraria tuberosa*, commonly known as Vidarikand in India, is an important plant used in traditional medicine. However, there are at least three other botanical entities traded under the same name, namely *Ipomoea mauritiana*, *Adenia hondala* and *Cycas circinalis*. A DNA marker-based method has been developed for identifying *P. tuberosa*, which is the authentic vidari according to the Ayurvedic Pharmacopoeia of India. A putative 600 bp marker specific to *P. tuberosa* was identified using the RAPD technique. Further, the RAPD amplicon was converted to a sequence characterized amplified region (SCAR) marker. PCR using**

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**designed SCAR primers revealed the expected amplicon (320 bp) only in *P. tuberosa* and not in the other species, thus aiding in distinguishing the authentic *P. tuberosa* from its commonly used substitutes and adulterants. Authentication of medicinal raw drug identity and quality using molecular markers holds great promise in resolving issues of controversial identity and quality control, but has not yet been capitalized by the traditional medicine sector.**

**Keywords:** Authentication, *Pueraria tuberosa*, SCAR marker, traditional medicine, Vidarikand.

*PUERARIA TUBEROSA* (Roxb. ex. Willd.) DC. (Fabaceae), one of the important plants used in Indian medicine, is commonly known as Vidarikand<sup>1</sup>. The tuberous roots of *P. tuberosa* are used to relieve symptoms of dysmenorrhoea, dysfunctional uterine bleeding and menopausal syndrome<sup>2</sup>. It possesses spasmolytic, anti-inflammatory<sup>2</sup>, anti-implantation<sup>3</sup>, anti-hyperglycaemic<sup>4</sup>, oestrogenic<sup>5</sup> and contraceptive<sup>6</sup> properties. Studies have been conducted on the chemistry and therapeutic effect of various parts of the plant. Phyto-compounds like  $\beta$ -sitositreol, stigmastrol, daidzein, puerarin<sup>7</sup>, puerarone and coumestan<sup>8</sup>, isoflavone C-glycoside-4,6-diacetyl-puerarin (root)<sup>7</sup>, pterocarpin-tuberosin (roots and tubers)<sup>9</sup>, puetuberosanol<sup>10</sup> and hydroxytuberosone<sup>11</sup> have been isolated and characterized from the species.

At least three other species are also sold as Vidarikand, including tubers of *Ipomoea mauritiana* Jacq. (Convolvulaceae), *Adenia hondala* (Gaertn.) W. J. de Wilde (Passifloraceae) and the pith of *Cycas circinalis* L. (Cycadaceae)<sup>2</sup>. To the untrained eye, the tubers appear similar, making authentication difficult and providing scope for adulteration, thus compromising quality.

Molecular technology is increasingly becoming popular as a powerful tool for unambiguous raw-drug authentication. The Chinese are making significant strides in this regard in traditional Chinese medicine. PCR-based methods, including random amplified polymorphic DNA (RAPD), have been used for authentication of medicinal plant materials. However, RAPD markers are difficult to reproduce and are therefore preferentially converted to more specific sequence characterized amplified region (SCAR) markers. These markers have been used for authentication of medicinal plant species of *Panax ginseng*<sup>12</sup>, *Piper longum* L.<sup>13</sup>, *Artemisia princeps* and *A. argyi*<sup>14</sup>, *Phyllanthus emblica*<sup>15</sup> and other commercially important timbers like bamboo<sup>16</sup> and strawberry<sup>17</sup>.

In the present study, DNA fingerprints of *P. tuberosa* and its common substitutes, namely *I. mauritiana*, *A. hondala* and *C. circinalis* were developed using RAPD-PCR. A unique polymorphic band in *P. tuberosa* was identified. Subsequently, the polymorphic amplicon was sequenced and sequence-specific SCAR primers designed. Testing of the SCAR primers using genomic

DNA of *P. tuberosa* and its substitutes confirmed amplification only in *P. tuberosa* samples, but not in its substitutes.

Authentic raw drugs were collected from Kerala, Maharashtra and Karnataka (India), by qualified field botanists at the FRLHT, Bangalore. The samples were deposited in the Herbarium and Raw Drug Repository (FRLHT). The raw drugs were dried at room temperature, chopped into small pieces and powdered in liquid nitrogen using pestle and mortar. DNA was extracted from the powdered samples by homogenizing them in CTAB extraction buffer, according to the protocol of Milligan<sup>18</sup>. The extracted DNA was treated with 5  $\mu$ l of RNAase (10 mg/ml) to remove any contaminating RNA. Purity of DNA was checked using UV-VIS spectrophotometer (Shimadzu), by calculating the  $A_{260}/A_{280}$  ratio as mentioned by Sambrook and Russell<sup>19</sup>.

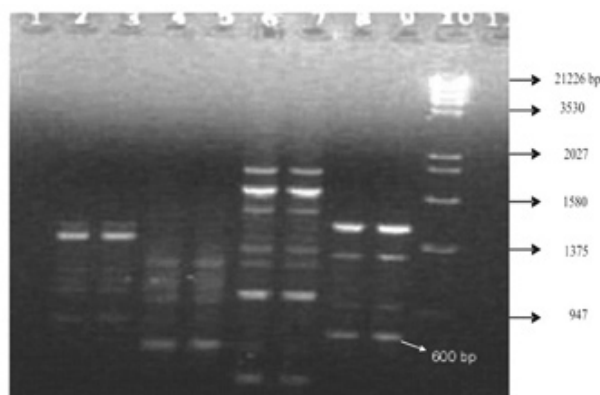
Polymerase chain reaction (PCR) was performed by adding 25 ng of plant DNA, 10 mM dNTP mix, 50 pM of primer, 2.5  $\mu$ l of 10 $\times$  PCR buffer, 2.5 mM of MgCl<sub>2</sub>, 2.0 U of *Taq* DNA polymerase (Genei, Sanmar Chemicals, India) and made up to 25  $\mu$ l with distilled water. The tube was placed in a thermocycler (Gradient PCR, Eppendorf) and PCR was performed as follows: 94°C for 3 min, 40 cycles of 94°C for 30 s, 36°C for 30 s, 72°C for 1 min and a final extension of 72°C for 10 min. The PCR products were resolved along with a  $\lambda$ -DNA/*EcoRI/HindIII* digest marker (Genei, Sanmar Chemicals), on a 1.2% agarose gel. The DNA was stained with ethidium bromide and photographed under UV transilluminator. OPA kit (OPA 1–20, Operon Technologies, USA) was used to screen the plant samples for polymorphism. All the 20 primers used for screening produced distinct reproducible amplification profiles for the screened DNAs of *P. tuberosa*, *I. mauritiana*, *C. circinalis* and *A. hondala*. Among these, OPA 08 revealed a polymorphic band for *P. tuberosa* species and was selected. Primer OPA 08 consistently amplified an intense band of around 600 bp in all the *P. tuberosa* species, which was absent in *I. mauritiana*, *C. circinalis* and *A. hondala* (Figure 1). The banding pattern was consistent when tested on at least four different accessions of *P. tuberosa* and repeated at least three different times each (Figure 2).

The 600 bp polymorphic band was gel-purified following the manufacturer's instructions (QIAquick Gel Extraction Kit, Qiagen, USA), cloned into pDRIVE cloning vector and transformed into Qiagen EZ competent cells following the supplier's instructions (Qiagen PCR Cloning Plus Kit, Qiagen). The recombinant construct was sequenced on an ABI 310 automated sequencer (Applied Biosystems, USA) using a DyeDeoxy™ Terminator Cycle Sequencing kit, as recommended by the manufacturer (Applied Biosystems, USA). The vector-specific promoter primers SP6 and T7 (Bioserve Biotechnologies Pvt Ltd, Hyderabad) were used for sequencing. The length of the polymorphic band sequence obtained was 600 bp with

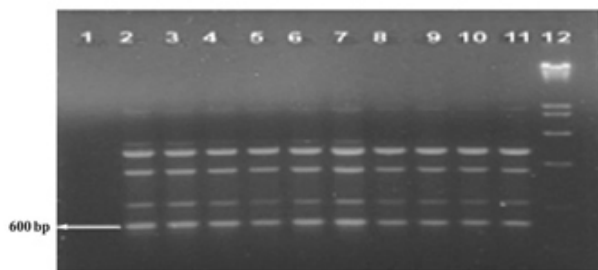
## RESEARCH COMMUNICATIONS

G + C content of 47.5% (A : 154; T : 161; G : 127; C : 158). The first ten nucleotides of the sequence obtained matched completely with the corresponding RAPD primer OPA 08 used (OPA 08 GTGACGTAGG; Figure 3). The DNA sequence was deposited with the National Center for Biotechnology Database, USA (NCBI Accession no: EU031785). Homology searches were performed within the non-redundant database of GenBank using the BLAST algorithm at <http://www.ncbi.nlm.nih.gov/BLAST/> of NCBI, with the program BLASTX. BLAST results revealed that the sequence did not have any homology with any known nucleotide sequences.

Based on the sequences of the RAPD amplicon, a pair of SCAR oligonucleotide primers (Pt 1FP; Pt 1RP) which could amplify approximately 320 bp of the genomic *P. tuberosa* DNA was designed. Pt 1FP (22-mer) was designed as the forward primer and Pt 1RP (26-mer) as reverse primer (Table 1). The sequences were custom-synthesized from Bioserve Biotechnologies Pvt Ltd (Hyderabad). The pair of SCAR primers was used for PCR amplifications of genomic DNA from *P. tuberosa*, *I. mauritiana*, *A. hondala* and *C. circinalis*. Thermal cy-



**Figure 1.** RAPD-PCR of species used as Vidari with OPA 08 primer. The 600 bp polymorphic band is indicated in lanes 8 and 9 of *Pueraria tuberosa*. Lanes 2, 3, *Adenia hondala*; lanes 4, 5, *Cycas circinalis*; lanes 6, 7, *Ipomoea mauritiana*; lanes 8, 9, *P. tuberosa*, and lane 10, marker,  $\lambda$ -DNA (*EcoRI/HindIII* digest).



**Figure 2.** Selected primer OPA 8 showing unique DNA fingerprints in different samples of *P. tuberosa*. Lanes 2–11, DNA samples of *P. tuberosa*, and lane 12,  $\lambda$ -DNA (*EcoRI/HindIII*) marker.

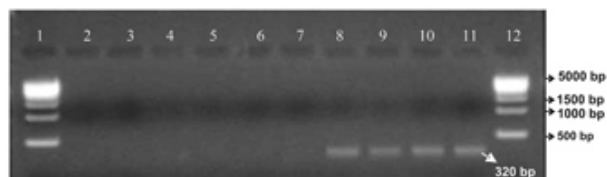
cling conditions for amplification using SCAR primers were optimized as: 95°C for 4 min, 35 cycles of 95°C for 45 s, 58°C for 45 s and 72°C for 60 s and a final extension at 72°C for 10 min. A single, distinct and bright band of 320 bp was obtained with DNA isolated from all the samples of *P. tuberosa* and no non-specific amplification was observed in *I. mauritiana*, *C. circinalis* and *A. hondala* (Figure 4). Reduction of the annealing temperatures did not generate any fragment other than the SCAR marker, confirming the ruggedness and specificity of the SCAR primer for *P. tuberosa*.

In essence, we have developed the SCAR marker to authenticate the identity of *P. tuberosa*. RAPD analysis can reveal a high degree of polymorphism and does not require prior DNA sequence information of the species; it is also easy to perform. Therefore, various researchers have explored its application for authentication of traditional Chinese medicines (TCM) like ginseng<sup>20</sup>, Echinacea<sup>21</sup> and Atractylodes<sup>22</sup>. The Chinese have forged ahead in using various DNA-based technologies to characterize plant and animal raw drugs used in TCM<sup>23</sup>. In the current study, RAPD analysis yielded significant genetic polymorphism among *P. tuberosa* and its substitutes (Figure 1). Also, the SCAR primers produced a single band of 320 bp only in *P. tuberosa*, making the marker more specific and dependable compared to just a RAPD marker (Figure 4). The SCAR primers could thus be used for authentication

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1  GTGACGTAGGCTTCTAATGGGTGATCGCATTCCGCAACTGTAACGGATTACCCCTAGGTG
   OPA 08
61  TTCTTCCCTCTGGTCCCTCGCAACCAGCAAGTTCTCCTTCATCGGACAATCTCGTGCCT
121 GAGGAGACTCTTACATACAAATGAAAGAGCCAGAGTCCGCCAGGCTTCTTTGCTGTCC
   Pt 1 FP
181 ATCTGTGTGAGAGGCATTCTTCCACTTCTGGCCGGTTTCTTCTTGTCAATTTCAACCCC
241 TCTGTTGTGCACTAAGAACTTTAGGAAGTTTCTGCTGTGTACGCAAAATGCACATTTTTA
301 CGGGTTCATCTTCAAGCCATGTTCTCGCGTCAAACTGCTGTGAGATCAGACAAATAA
361 CCCGAAGAAACAGGAAAGACTTCACAACACTACATCGTCAATATACACCACCTCTACGATG
421 TTGACAATAAAGTAATGGGAAGATGAGGTTTCATTGCTTCGCTGGTGGTAACTATAGCGTT
   Pt 1 RP
481 ATTTCAGACCGAAGGGCATCACCTGCGCAGCAGAATAGTCCGTCCAAACAGCCCGGACAAC
541 GGAACGCTGTTTTTGCCCTCTCTTTTGGAGATGAAAAAAGGTTTATACCCCTAGCTCAC
   OPA 08
    
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**Figure 3.** Sequence of 600 bp polymorphic RAPD amplicon from *P. tuberosa* using primer OPA 08. The OPA 08, Pt 1FP (forward primer) and Pt 1RP (reverse primer) sequences are highlighted.



**Figure 4.** PCR amplification of *A. hondala*, *C. circinalis*, *I. mauritiana* and *P. tuberosa* using Pt 1FP and Pt 1RP. Amplification of specific SCAR marker can be observed only in *P. tuberosa* samples. Lanes 1, 12, 500 bp marker; lanes 2, 3, *A. hondala*; lanes 4, 5, *C. circinalis*; lanes 6, 7, *I. mauritiana* and lanes 8–11, *P. tuberosa*.

**Table 1.** *Pueraria tuberosa*-specific SCAR primer designed from the sequenced 600 bp RAPD amplicon

RAPD primer	SCAR primer	Number of base pairs	Sequence (5'-3')	G + C content (%)	Annealing temperature (°C)
OPA 08	Pt 1 FP	22	AGA GCC AGA GTC CGC CAG GCC T	68	62
	Pt 1 RP	26	ACC AGC GAG CAA TGA ACC TCA TCT TC	50	60

of *P. tuberosa* species and to distinguish it from its substitutes. A fraction of the quantities used in other conventional pharmacognostic techniques is required for SCAR testing and more number of samples can be tested within a short time. Such techniques are required not only to complement conventional parameters in creating the passport data of medicinal raw drugs, but also for routine quality control in the herbal industry and in commercial and Government testing laboratories. Contrary to the common understanding among the traditional medicine sector in India, routine use of DNA technology as a quality control tool will not be more expensive than conventional HPTLC/HPLC methods, once the protocol and markers are available.

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