Development of Randomly Amplified Polymorphic DNA-Sequence Characterized Amplified Region Marker for Identification of *Apocynum venetum* LINN. from *A. pictum* SCHRENK

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Apocynum venetum LINN. is an important Chinese crude drug, and its sibling species *A. pictum* SCHRENK is a confusable herb which is similar to it. The purpose of this study is to develop DNA molecular markers to distinguish *A. venetum* from *A. pictum* through the combinative technologies of bulked segregate analysis (BSA) and randomly amplified polymorphic DNA (RAPD). Two putative markers B08-407 and B03-1368 specific for *A. venetum* were identified and sequenced. Based on the sequence information, two pairs of primers were designed and synthesized for sequence characterized amplified region (SCAR) markers. But only one primer pair, B03-1368, produced a clear SCAR band in all samples of *A. venetum* and not in *A. pictum*. This SCAR marker was found useful for rapid identification of *A. venetum* from *A. pictum*.

Key words *Apocynum venetum*; *Apocynum pictum*; identification; sequence characterized amplified region; randomly amplified polymorphic DNA; bulked segregate analysis

Apocynum venetum LINN. is a Chinese specific wild plant resource and belongs to *Apocynum* of Apocynaceae. *A. venetum* has been treated as medicine for more than a thousand years, the components such as flavanoid contained in its leaves have the effects of preventing and relieving high blood pressure,^{1,2)} hyperlipemia,³⁾ anxietas,⁴⁾ liver injury,⁵⁾ asthma and tracheitis⁶⁾ etc., which is recorded by “Chinese Pharmacopoeia”.⁷⁾ *A. pictum* SCHRENK has a similar appearance of *A. venetum*,⁸⁾ but is mainly applied in industries as fibres for spinning and papermaking etc. However, both *A. venetum* and *A. pictum* are known as “Luobuma” in Chinese people, and in recent years, *A. venetum* was in the verge of extinction for over-exploitation, only *A. pictum* has large areas of pure colonia.⁹⁾ It was common that *A. venetum* was replaced by *A. pictum* for medicinal, tea and tobacco use, which led to the confusion of the medicine and health products markets.

Former researchers have done some identification studies on the morphologic characteristics¹⁰⁾ and chemical compositions¹¹⁾ of the leaves of *A. venetum* and *A. pictum*. However, *A. venetum* distributes in a large area of about 14 provinces north of the Yangtze River east to the coast of the Yellow Sea and west to Xinjiang Uygur Autonomous Region, and *A. pictum* mainly grows on barren salt lick, dry river valley or Gobi desert in 3 provinces of Xinjiang, Gansu and Qinghai. The tremendous differences of the natural conditions, including the conditions of soil, water and atmosphere, lead to the great differences in appearance, structure and chemical compositions of leaves of *A. venetum* and *A. pictum*, especially *A. venetum*.^{12,13)} In addition, the appearance, structure and chemical compositions of leaves were changing with the changes of growth stage¹³⁾ and the appearance and structure of the leaves were destroyed after the leaves being made into some goods, for instance the powdered goods (Table 2). The identification methods based on morphologic characteristics and chemical compositions were not workable. With the development of molecular biology and the innovation of related technologies, the identification of plants and medicinal sub-

stances etc. on the DNA molecular level has become a feasible and widely used method.¹⁴⁾ The author had tried to use the DNA direct sequencing method to identify *A. venetum* from *A. pictum*,¹⁵⁾ but the sequencing cost was comparatively high and the sequencing time was comparatively long, which was adverse to rapid identification. While randomly amplified polymorphic DNA-sequence characterized amplified region (RAPD-SCAR) which was developed on the basis of RAPD had been successfully applied to the identification of medicinal plants such as *Phyllanthus emblica*¹⁴⁾ and *Cenchrus ciliaris*¹⁷⁾ etc.

In this paper, combined technologies of RAPD and bulked segregate analysis (BSA) were first used to amplify samples of *A. venetum* and *A. pictum* from different areas of China and to find the RAPD differential bands between *A. venetum* and *A. pictum*. Then the differential fragments were cloned and sequenced. According to the sequences, specific identification primers were designed and transformed into SCAR markers which could distinguish *A. venetum* from *A. pictum* quickly and exactly.

MATERIALS AND METHODS

Materials All the plant materials were collected by the Chinese Luobuma resource scientific investigation team from various regions of China in 2005 and the concrete information was shown in Table 1. Under natural conditions, *A. venetum* and *A. pictum* reproduce themselves by asexual reproduction. In order to avoid collecting samples with the same genetic composition, the distances between different samples were at least 1 km or more. Leaves were dried by silica gel and identified by Xiao Zhengchun from Nanjing Institute of Comprehensive Utilization of Wild Plants. Voucher specimens (collection No. from 200535 to 2005109) were deposited at the herbarium of Nanjing Institute of Comprehensive Utilization of Wild Plants for further reference. Six Luobuma goods samples (collection No. from 2005110 to

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Table 1. Plant Materials

Species	Sample codes	Origin/collector	Number of samples	Voucher number	Collection date
<i>Apocynum venetum</i> LINN.	L1	Altay, Xinjiang/Gu G.	5	200545—200549	2005—7—18
	L2	Korla, Xinjiang/Zhang W.	5	200535—200539	2005—7—14
	L3	Tongliaoza Qi, Inner Mongolia/Gong Z.	5	200575—200579	2005—8—15
	L4	Yumen, Gansu/Gong Z.	5	200580—200584	2005—8—30
	L5	Yinchuan, Ningxia/Xian X.	5	200565—200569	2005—8—5
	L6	Xingping, Shanxi/Qian X.	5	200560—200564	2005—7—27
	L7	Sanmenxia, Henan/Qian X.	5	200595—200599	2005—9—8
	L8	Dongtai, Jiangsu/Zhang W.	5	2005105—2005109	2005—9—15
	L9	Changling, Jilin/Gong Z.	5	200570—200574	2005—8—10
	L10	Xiaoxian, Anhui/Lu Z.	5	2005100—2005104	2005—9—12
<i>A. pictum</i> SCHRENK	B1	Bachu, Xinjiang/Gu G.	5	200550—200554	2005—7—20
	B2	Korla, Xinjiang/Zhang W.	5	200540—200544	2005—7—14
	B3	Yumen, Gansu Gong Z.	5	200585—200589	2005—8—30
	B4	Dunhuang, Gansu/Gu G.	5	200555—200559	2005—7—26
	B5	Gomud, Qinghai/Gu G.	5	200590—200594	2005—9—2

All the materials were collected by the Chinese Luobuma resource scientific investigation team in 2005 and identified by Xiao Zhengchun.

Table 2. Luobuma Goods Samples

No.	Trade name	Raw material/origin	Product character	Manufacturer	Voucher number
1	"Luto" Apocynum tea	<i>A. pictum</i> /Xinjiang	Enveloped	Xinjiang Bachu County Apocynum Tea Plant	2005110
2	"Big flower" Luobuma tea	<i>A. pictum</i> /Xinjiang	Enveloped	Xinjiang Yuli Jiangbao Comprehensive Exploitation of Luobuma Co., Ltd.	2005111
3	"Tonghui" Luobuma tea	<i>A. venetum</i> /Xinjiang	Powder	Inner Mongolia Triumph Industrial Co., Ltd.	2005112
4	"Jinran" Lobm tea	<i>A. venetum</i> /Shanxi	Crimple and curled	Shanxi Runhua Tea Co., Ltd.	2005113
5	"Baisui" Luobuma tea	<i>A. venetum</i> /Xinjiang	Enveloped	Xinjiang Korla Alham Luobuma Plant	2005114
6	"Niyaren" Luobuma tea	<i>A. venetum</i> /Xinjiang	Powder	Xinjiang Green Health Luobuma Co., Ltd.	2005115

All the goods samples were purchased by the Chinese Luobuma resource scientific investigation team in 2005 directly from the manufacturer and the identification of the raw materials was according to the combined information of packing indication, original place of raw materials and the fresh leaves in factories.

2005115) were purchased by the Chinese Luobuma resource scientific investigation team in 2005 directly from the manufacturer and the identification of the raw materials was according to the combined information of packing indication, original place of raw material and the fresh leaves in factories. Their concrete information was shown in Table 2.

DNA Isolation DNA was extracted with a Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, U.S.A.). DNA extractions followed manufacturer's protocols with the modification of buffer AP1 lysis conditions by the addition of papain (Sigma) to the final concentration of 50 mg/ml, and incubation at 37 °C for 12 h on a rocking platform.

Construction of Gene Pool Two gene pools were constructed by the sample genome DNA of *A. venetum* from 10 regions and *A. pictum* from 5 regions, and the template DNA concentration of the two gene pools was 20 ng/μl.

RAPD-Polymerase Chain Reaction (PCR) The PCR amplification was performed in a 25 μl reaction system. A reaction tube contained 30 ng of DNA, 1.5 U of *Taq* DNA polymerase enzyme, 5 pmol of each deoxyribonucleotide triphosphate (dNTP), 1X *Taq* polymerase buffer with 2.5 mM of MgCl₂, and 10 pmol of each primer. The amplification was carried out in a DNA thermal cycler (Eppendorf, German) using following parameters: 94 °C for 5 min; 42 cycles at 94 °C for 45 s, 36 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 7 min. PCR products were subjected to agarose gel (1.5% [w/v]) electrophoresis in 0.5X TBE buffer, along with DNA ladders (Takara, Japan) as size markers. DNA was stained with ethidium bromide and photographed

under UV light (GDS-8000, UVP, U.S.A.).

Screening Strategy and Identification of Specific RAPD Amplicon Eighty random primers (Table 3) (Shanghai Sangon, Inc., China) were screened by RAPD for identification of specific marker. Each amplification of DNA on two pools or samples from 10 populations of *A. venetum* and 5 populations of *A. pictum* was performed with a single primer. The amplicons, which only existed in all samples of *A. venetum* and not existed in samples of *A. pictum* were identified.

Cloning and Sequencing of Specific RAPD Fragments The specific RAPD fragments were purified with a 3S Spin Agarose Gel DNA Purification Kit (Shanghai Shenergy Bio-color Inc., China), and cloned in pMD18-T vector (Takara, Japan). The plasmids harboring inserts of the expected size were extracted and sequenced partially at both ends using an automated sequencer (Prism 377, ABI, U.S.A.). The DNA sequences were submitted to GenBank (Accession numbers: ET030151 and ET030150).

Designing SCAR Primers and SCAR-PCR Two pairs of SCAR primers were designed from the sequence information of the specific RAPD fragments. They were used to amplify the two gene pools and the genome DNA of each sample of *A. venetum* and *A. pictum*. What contained in each reaction tube was similar to the above-mentioned. Thermal cycling conditions for the amplification were optimized as: 94 °C for 3 min; 29 cycles at 94 °C for 45 s, 59 °C for 30 s, and 72 °C for 90 s; and a final extension at 72 °C for 7 min. PCR amplification for authentication of commercial samples was carried out using the SCAR primer pair with the above-

Table 3. Names and Sequences of Random Primers

Primer	Sequence (5'→3')	Primer	Sequence (5'→3')
A01*	CAGGCCCTTC	C01	TTCGAGCCAG
A02*	TGCCGAGCTG	C02*	GTGAGGCGTC
A03	AGTCAGCCAC	C03	GTGAGGCGTC
A04	AATCGGGCTG	C04	CCGCATCTAC
A05	AGGGGTCTTG	C05	GATGACCGCC
A06	GGTCCCTGAC	C06*	GAACGGACTC
A07	GAAACGGGTG	C07	GTCCCGACGA
A08	GTGACGTAGG	C08*	TGGACCGGTG
A09*	GGGTAACGCC	C09*	CTCACCCTCC
A10*	GTGATCGCAG	C10*	TGTCTGGGTG
A11*	CAATCGCCGT	C11	AAAGCTGCGG
A12	TCGGCGATAG	C12*	TGTCATCCCC
A13	CAGCACCCAC	C13	AAGCCTCGTC
A14*	TCTGTGCTGG	C14	TGCGTGCTTG
A15*	TTCCGAACCC	C15*	GACGGATCAG
A16*	AGCCAGCGAA	C16*	CACACTCCAG
A17*	GACCGCTTGT	C17	TTCCCCCAG
A18*	AGGTGACCGT	C18	TGAGTGGGTG
A19	CAAACGTCGG	C19	GTGCGCAGCC
A20	GTTGCGATCC	C20	ACTTCGCCAC
B01*	GTTTCGCTCC	D01*	ACCGCGAAGG
B02*	TGATCCCTGG	D02	GGACCCAACC
B03*	CATCCCCCTG	D03*	GTGCGCGTCA
B04*	GGACTGGAGT	D04	TCTGGTGAGG
B05*	TGCGCCCTTC	D05	TGAGCGGACA
B06	TGCTCTGCCC	D06	ACCTGAACGG
B07	GGTGACGCAG	D07*	TTGGCACGGG
B08*	GTCCACACGG	D08	GTGTGCCCCA
B09	TGGGGGACTC	D09	CTCTGGAGAC
B10*	CTGCTGGGAC	D10	GGTCTACACC
B11*	GTAGACCCGT	D11	AGCGCCATTG
B12*	CCTTGACGCA	D12	CACCGTATCC
B13	TTCCCCCGCT	D13	GGGGTGACGA
B14	TCCGCTCTGG	D14	CTTCCCCAAG
B15*	GGAGGGTGTT	D15	CATCCGTGCT
B16	TTTGCCCGGA	D16*	AGGGCGTAAG
B17	AGGGAACGAG	D17	TTTCCACGGG
B18	CCACAGCAGT	D18*	GAGAGCCAAC
B19	ACCCCGAAG	D19*	CTGGGGACTT
B20	GGACCCCTAC	D20	ACCCGGTAC

They were purchased from Shanghai Sangon, Inc. of China. The asterisk represents the primers which produced clear differential bands.

mentioned thermal cycling conditions.

RESULTS

The gene pools of *A. venetum* and *A. pictum* were amplified and screened by 80 RAPD random primers. It was found that 34 random primers could generate clear differential bands between *A. venetum* and *A. pictum* (Table 3).

Then, the genome DNA of each *A. venetum* samples from 10 regions and each *A. pictum* samples from 5 regions were performed with RAPD-PCR amplification again by the selected 34 primers. Results showed that only primer B08 and B03 could generate clear and stable difference between all detected samples of *A. venetum* and *A. pictum*. Primer B08 could produce bands in all *A. venetum* samples near 450 bp, but not in any *A. pictum* sample. Because the RAPD pattern of different samples from the same region were very similar and the lane numbers in one electrophoresis blanket was limited, Fig. 1, together with Figs. 2 and 4, only showed the result from one randomly selected sample of every region. The band was recorded as B08-450 temporarily. Primer B03

Fig. 1. RAPD Pattern Amplified with B08 Primer

M: DNA marker; L1—L10: samples of *A. venetum*; B1—B5: samples of *A. pictum*. Their voucher numbers were 200545, 200538, 200576, 200583, 200566, 200563, 200597, 2005108, 200572, 2005103, 200552, 200542, 200588, 200557 and 200593, respectively. Their collection localities were showed in Table 1. The white arrow showed the different bands between *A. venetum* and *A. pictum*.

Fig. 2. RAPD Pattern Amplified with B03 Primer

M: DNA marker; L1—L10: samples of *A. venetum*; B1—B5: samples of *A. pictum*. Their voucher numbers were 200547, 200536, 200578, 200567, 200564, 200596, 2005106, 200574, 2005102, 200552, 200542, 200587, 200556 and 200593, respectively. Their collection localities were showed in Table 1. The white arrow showed the different bands between *A. venetum* and *A. pictum*.

Fig. 3. Optimization of PCR Amplification on Pools of *A. venetum* (L) and *A. pictum* (B) with B03-1368 SCAR Primer

M: DNA marker; L: pool of *A. venetum*; B: pool of *A. pictum*. Annealing temperature ranging from 56 to 60 °C.

Fig. 4. PCR Pattern of *A. venetum* (L) and *A. pictum* (B) Obtained with B03-1368 SCAR Primer

L1—L10: samples of *A. venetum*; B1—B5: samples of *A. pictum*. Their voucher numbers were 200545, 200536, 200578, 200581, 200569, 200564, 200597, 2005106, 200574, 2005103, 200553, 200543, 200589, 200557 and 200592, respectively. Their collection localities were shown in Table 1.

could produce bands in all *A. venetum* samples near 1500 bp, but not in any *A. pictum* sample (Fig. 2). The band was recorded as B03-1500 temporarily. B08-450 and B03-1500 were selected as the putative specific markers for *A. venetum*.

Full-length sequences of the two fragments were obtained by the cloning and sequencing of B08-450 and B03-1500, and the primal 10 base of the two sequences were exactly the

Table 4. Designed SCAR Primer Pairs and Sizes of SCAR-PCR Products

RAPD marker	GenBank accession number	SCAR primer sequence (5'→3')/size/bp	Size of SCAR marker/bp
B08-407	ET030151	F-gtccacacggcaggag/16 R-gtccacacgggttcc/16	407
B03-1368	ET030150	F-catccccctgcccc/14 R-catccccctgagtcag/17	1368

Fig. 5. PCR Amplification of Genomic DNA from 6 Commercial Samples of *A. venetum* and *A. pictum* with B03-1368 SCAR Primer

M: DNA marker; 1, 2: *A. pictum*; 3—6: *A. venetum*. Their concrete information was shown in Table 2.

same as the corresponding RAPD primer B08 and B03. Their GenBank accession numbers were ET030151 and ET030150, and their actual measured lengths were 407 bp and 1368 bp, respectively. So, B08-450 and B03-1500 were renamed as B08-407 and B03-1368.

According to the both ends sequences of segment B08-407 and B03-1368, two pairs of SCAR primers B08-407F/R and B03-1368F/R (Table 4) were designed and synthesized. Using the gene pools of *A. venetum* and *A. pictum* as templates, the identification feasibility of the two pairs of primers was detected with the reannealing temperature ranging from 50 to 60 °C. The amplification conditions were optimized simultaneously. When the B08-407 SCAR primer pairs were detected, both gene pools produced clear bands near 400 bp when the reannealing temperature ranging from 50 to 57 °C, but both produced no band when the temperature over 58 °C. When B03-1368 SCAR primer pairs were detected, the gene pool of *A. venetum* produced obvious band near the corresponding location of 1368 p with the reannealing temperature ranging from 56 to 59 °C, and at 59 °C, the band was the most clear and no mixed band existed, while the gene pool of *A. pictum* produced no band within the above temperature range (Fig. 3). Therefore, B08-407 SCAR primer was invalid, and the best reannealing temperature for B03-1368 SCAR primer was 59 °C.

Each genome DNA of all detected samples of *A. venetum* and *A. pictum* was amplified under optimized conditions by the B03-1368 SCAR primer pair. The results showed that all *A. venetum* samples had a clear and bright band at 1368 bp, while all *A. pictum* samples had no amplified band at that site (Fig. 4).

The genome DNA of 6 commercial samples of *A. venetum* and *A. pictum* were amplified with B03-1368 SCAR primer pair further. Four *A. venetum* samples could repeatedly amplify clear bands near 1368 bp, while 2 *A. pictum* samples had no clear band (Fig. 5).

DISCUSSION

A. venetum and *A. pictum* are two different species of *Apocynum* distributed in China. There is no definite differ-

ence between the leaves of the two plants, and the morphosis of leaves is also different in different growth stages and different environments.¹³⁾ What's more, the morphosis of the leaves is destroyed when the leaves are processed into products. Therefore, the identification of *A. venetum* and *A. pictum* is always a problem that people hope to solve. *A. venetum* and *A. pictum* could be accurately identified by the direct determination and comparison of the sequences in the *trnL* intron and *trnL*-F non-coding region,¹⁵⁾ but the cost is comparatively high and the sequencing time is too long, which is adverse to rapid identification. Restriction enzyme recognition sites in the two regions from *A. venetum* and *A. pictum* are exactly the same, and PCR-RFLP method cannot be used to distinguish them. Furthermore, we have tried to design specific primers in accordance with the sequence differences of these two regions between them. But too narrow range of annealing temperature means little practical application.¹⁵⁾

RAPD analysis can reveal high degree of polymorphism which does not require prior DNA sequence information of the species and is easy to perform. Various researchers have explored its application for authentication of traditional medicines *Phyllanthus emblica*,¹⁴⁾ *Cenchrus ciliaris*¹⁷⁾ etc.

The combination of BSA and RAPD methods is usually used to find the molecular markers which are linked to target gene.¹⁸⁾ In order to facilitate the screening of primers, methods of BSA and RAPD were combined and the intraspecies genetic differences of different regions and different individuals were ignored in this study. Thirty-four primers which could produced obvious difference between two gene pools were picked out from 80 random primers quickly. This saved much time for preliminary screening of primers. Then, the selected 34 primers were used to amplify the samples from different regions of China, and only two primers B03 and B08 could generate clear and different bands between all detected samples of *A. venetum* and *A. pictum*.

Because RAPD random primer has a length of only 10 bp, the reproducibility of RAPD reaction is not so good and the analysis is relatively complex.¹⁹⁾ But in SCAR, the primers are completely complementary with the nucleotide sequence of the species waiting for identification and the extended primers could amplify specific genome fragment under strict conditions, which make SCAR markers more specific and reliable than RAPD markers²⁰⁾ (Fig. 3). Two pairs of SCAR primers were designed according to the sequences of fragments B08-407 and B03-1368, but only B03-1368 SCAR primer could amplify the band with corresponding length in all natural and commercial *A. venetum* samples, while no such band could be amplified in all *A. pictum* samples (Figs. 4, 5). Therefore, through this study, we have developed a RAPD-SCAR marker for identification of *A. venetum* from *A. pictum*.

A. venetum is widely distributed in large areas of China. *A. venetum* from different regions has a certain difference in phenotypes and curative effects. How to find out the *A. vene-*

tum resource with high quality from so many types of *A. venetum* and to develop the molecular markers is our ongoing work.

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