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Original Article

Identification of SCAR markers for genetic authentication of *Dendrobium nobile* Lindl.

Identificação de marcadores SCAR para autenticação genética de *Dendrobium nobile* Lindl.

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Abstract

Dendrobium nobile Lindl. is an orcid plant with important medicinal values. This is a colourful houseplant, and also a popular herb in traditional Chinese medicine (TCM). The variants of this plant from different geographic regions might be high, and in this study, we aimed to develop specific sequence characterized amplified region (SCAR) markers for the identification of specific variant of this plant. Different cultivars of *D. nobile* were collected from nine different places of China, and one cultivar from Myanmar. DNA materials were extracted from the plant samples, random amplified polymorphic DNA (RAPD) were developed, cloned and sequenced for the development of SCAR markers. We have developed four SCAR markers, which are specific to the cultivar from Luzhou China, and clearly distinguishable (genetically) from other cultivars. These SCAR markers are deposited in GenBank (accession number MZ417502, MZ484089, MZ417504 and MZ417505). Four SCAR markers for *D. nobile* are effective molecular technique to genetically identify the different cultivars or species, and this method is applicable for genetic characterization and identification of other plant species too.

Keywords: *Dendrobium nobile* Lindl, genetic authentication, random amplified polymorphic DNA, sequence characterized amplified region.

Resumo

Dendrobium nobile Lindl. é uma orquídea com importantes valores medicinais. Esta é uma colorida planta doméstica e também uma erva popular na Medicina Tradicional Chinesa (MTC). As variantes desta planta de diferentes regiões geográficas podem ser altas, e neste estudo, nosso objetivo foi desenvolver marcadores de região amplificada de sequência caracterizada (in English, *Sequence Characterized Amplified Region* (SCAR)) para a identificação de variante específica desta planta. Diferentes cultivares de *D. nobile* foram coletadas de nove locais diferentes da China e uma cultivar de Mianmar. Materiais de DNA foram extraídos das amostras de plantas, em que a Amplificação Aleatória de DNA Polimórfico (in English, *Random Amplified Polymorphism DNA* (RAPD)) foi desenvolvida, clonada e sequenciada para o desenvolvimento de marcadores SCAR. Desenvolvemos quatro marcadores SCAR, que são específicos para a cultivar de Luzhou na China e claramente distinguíveis (geneticamente) de outras cultivares. Esses marcadores SCAR estão depositados no GenBank (números de acesso MZ417502, MZ484089, MZ417504 e MZ417505). Quatro marcadores SCAR para *D. nobile* compreendem técnicas moleculares eficazes para identificar geneticamente as diferentes cultivares ou espécies, e este método é aplicável para caracterização genética e identificação de outras espécies de plantas também.

Palavras-chave: *Dendrobium nobile* Lindl, autenticação genética, amplificação aleatória de DNA polimórfico, região amplificada de sequência caracterizada.

1. Introduction

Dendrobium nobile Lindl., belonging to the Orchidaceae family, commonly known as noble dendrobium, is a colourful houseplant, but it is also a popular herb practised in traditional Chinese medicine (TCM) (Flora of China, 2009). This is native to a wide range of region worldwide, including Southern China, South Asia, Indohina, Hawaii, New Zealand (Plants of the World Online, 2021). Due to its notable place in TCM, this plant has gained some interest among medicinal plant researchers and alternative medicine practitioners in last few decades. Active ingredients from this herb is mainly effective against different neuronal and cognitive diseases, as it is known for its potential effects on prevention of aging, because of its ability to scavenge oxidative free radicals, delaying

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the impairment of DNA impairment and altering DNA methylation (Nie et al., 2020). Some alkaloids from noble dendrobium was found to be effective against neuronal damage and aging as well as aging-related cognitive deficits in SAMP8 mice (Lv et al., 2020). Polysaccharides from noble dendrobium was found to protect the neurons and brain from hypoxic damage in mouse model with ischemic stroke by downregulating miR-134 and activating MCL-1 (Liu et al., 2021). Some polysaccharides have antiviral potential too (Li et al., 2020). Noble dendrobium powder has been tested clinically against metabolic syndrome (MS), and found potentially effective in certain patients as alternative therapy (Zhang et al., 2021). This herb has also been known for its potential against tumors, hyperlipidemia, hyperglycemia among diseases (Nie et al., 2020).

Although its rich history of usage in TCM, proper genetic characterization of noble dendrobium has not been performed. Genetic characterization is very important for protective species and biodiversity. With the recent developments in molecular biotechnology, different molecular biological tools are being employed for the genetic identification, characterization and authentication of different species of living organisms, including bacteria, fungi, plants and animals, for example, Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), Inter-Simple Sequence Repeat (ISSR), etc. (Williams et al., 1990; Fu et al., 2013; Khan et al., 2016). A very specific technique, named Sequence Characterized Amplified Region (SCAR) analysis, based on RAPD sequencing, has been used potentially to develop specific genetic markers of species (Fu et al., 2015, 2017; Liu et al., 2020), and these SCAR markers are very stable and reliable for high level authenticity of genetic characterization of target species. Here in this study, we have developed four SCAR markers, which can specifically authenticate the variants genetically.

2. Material and Methods

2.1. DNA preparation from noble dendrobium samples

Ten *Dendrobium nobile* Lindl. samples and one sample of the species *Dendrobium officinale* Kimura et Migo were collected from different geographic regions of China (Table 1, Figure 1). The genomic DNA of these samples were extracted by using Cetyl Trimethyl Ammonium Bromide (CTAB) method (slightly modified) (Fu et al., 2013). Shortly, the leaves were grinded and fixed with fixing solution (consisting 2-Hydroxy-1-ethanethiol, chloroform, PVP without liquid nitrogen), which was further grinded into small piece with silica for DNA extraction or isolation. DNA quality was checked by agarose gel electrophoresis (0.8%) and spectrophotometry (Fu, 2012). DNA samples were then further adjusted with 10 ng/µl, and for next use (suppose for PCR), was stored at -20°C.

2.2. Amplification (RAPD)

The RAPD primers used for the amplification of DNA samples were as follows: M9: TCTTGCGGA; M19: CCTTCAGGCA; N1: CTCACGTTGG; N2: ACCAGGGGCA,

Table 1. Sources of SCAR samples.

Sample	Common name	Sources	No.
LB	*D. nobile*	Laibin, Guangxi	001
YC	*D. nobile*	Yichang, HuBei	002
LZ	*D. nobile*	Luzhou, Sichuan	003
GJ	*D. nobile*	Gejiu, YunNan	004
NJ	*D. nobile*	Nujiang, YunNan	005
GZ	*D. nobile*	Ganzhou, JiangXi	006
WS	*D. nobile*	Wenshan, YunNan	007
JS	*D. nobile*	Jianshui, YunNan	008
ZZ	*D. nobile*	Zhangzhou, Fujian	009
MD	*D. nobile*	The Republic of the Union of Myanmar	010
TP	*D. officinale* Kimura et Migo	Liuan, AnHui	011

and amplification reaction was performed using Tiangen reagents (Beijing, China) following the protocols provided by the manufacturer. The contents of the 10 µl PCR reaction system was as follows: genomic DNA (1.5 µl), 2×Taq PCR MasterMix (5 µl), 2.5 µM primers (1 µl) and ddH_2O (2.5 µl). PCR reaction was executed in a thermocycler "Applied Biosystems Veriti® 96-Well Thermal Cycler" (Life Technology, USA).The reaction steps were as follows: (i) primary denaturation at 95°C (90s), (ii) 40 cycles of regular denaturation at 94°C (40s), annealing to extension adjusted to 0.125°C/s (5% RAMP rate) at 36°C (60s), and extension at 72°C (90s), (iii) final extension at 72°C (5m). The products of PCR reaction were then separated by electrophoresis on agarose gel (1.5%).

2.3. Cloning and identification of positive RAPD fragments

Two bright bands were chosen from the agarose gel, cut then purified by using TIANgel Mini DNA Purification Kit (DP209, TIANGEN, Beijing, China). The purified DNA fragments were then ligated into VT202pGM-T vector by AT cloning, which was then transformed into *E. coli* competent cells (DH5α). The recombinant clones were then cultured on Luria broth (LB) agar plates, containing ampicillin (100 µg/µl), IPTG (160 µg) and X-gal (40 mg).Culture plates were incubated for overnight at 37°C. White colonies were screened from the plates using the blue white screening method. From the white colonies, the right inserts were verified by PCR with SP6/T7 primers. Primer sequence were as follows: SP6, 5'-ATTTAGGTGACACTATAGAA-3'; T7, 5'-TAATACGACTCACTATAGGG-3'. PCR products were separated to get results on agarose gel electrophoresis (1.5%).

2.4. DNA sequencing and bioinformatics analysis

Sanger Sequencing method, using SP6 of T-vector sequencing primers, sequenced the positive clones. In search of the novelty of cloned RAPD fragments, we

Figure 1. The localities of samples *D. nobile* cultivars from different regions in China. Spots in black indicate cities and lines in light blue indicate the Yellow River (up) and the Yangtze River (down).

performed the homology search of the sequenced DNA clones using BLAST (NCBI online program; available here: http://www.ncbi.nlm.nih.gov/BLAST/) with various species in GenBank.

2.5. SCAR primers

SCAR primer pairs were designed by using cloned RAPD fragment's sequence. The primer sequences are presented in Table 2, which were designed by using online program Primer 3 (available at http://bioinfo.ut.ee/primer3-0.4.0/primer3/).

2.6. SCAR marker development

PCR amplifications were performed to develop stable and specific SCAR markers, from 21 DNA samples. The origin of DNA samples are listed in Table 1: one sample of *Lyciumbarbarum*L., *Litchi chinensis* Sonn., *Ginkgo biloba* L., *Canarium album* (Lour.) Raeusch., *Lonicera japonica* Thunb., Mentha canadensis Linnaeus., *Penthorum chinense* Pursh, Angelica sinensis, *Gardenia jasminoides,* Ganoderma lucidum (Leysser Fr.) Karst. In 10 µl of PCR reaction system, the contents were as follows: genomic DNA (1.5 µl), 2×Taq PCR MasterMix (5 µl), 2.5 µM primers (1 µl) and ddH_2O (2.5 µl). PCR reaction was executed in a thermocycler "Applied Biosystems Veriti® 96-Well Thermal Cycler" (Life Technology, USA). The PCR reaction steps were as follows: (i) primary denaturation at 95°C (90s), (ii) 33 cycles of regular denaturation at 94°C (40s), annealing at 60°C (30s) and extension at 72°C (40s), (iii) final extension at 72°C (5m). The products of PCR reaction were then separated by electrophoresis on agarose gel (1.8%), and visualized by ethidium bromide staining, and ChemiDoc XRS (Bio-Rad, USA) documented the images.

3. Results

3.1. Development of RAPD-fragments and their cloning

For improved RAPD amplification, four primers, termed as M9, M19, N1, N2 have been used and PCR results are presented in Figure 2 (black arrows show the bands labelled with desired primers). Those bands were then cut and purified from gel, and purified PCR products were then ligated into pGEM -T vector.

Table 2. Sequences of SCAR primers, PCR product size and PCR condition.

SCAR	5'-primer	Sequence (5'-3')	3'-primer	Sequence (3'-5')	Size (bp)	Tm (°C)
M19-45	M19-45L	AGGTGCCATGGAGAGTCTTG	M19-45R	CGCATCCAAAGATGGAAGAT	365	60
M9-21	M9-21L	TGCAGCCCTCCTCAAACTTT	M9-21R	TCAAAATGCAAACGGTCAAG	304	60
N1-12	N1-12L	TCGAGATATTCCCAGCGTTC	N1-12R	GTCAGTACGTCGGCGGTTAT	337	60
N2-11	N2-11L	ATTACCCTGCCATAGCGTTG	N2-11R	CCACGTAGCATCACACCATC	215	60

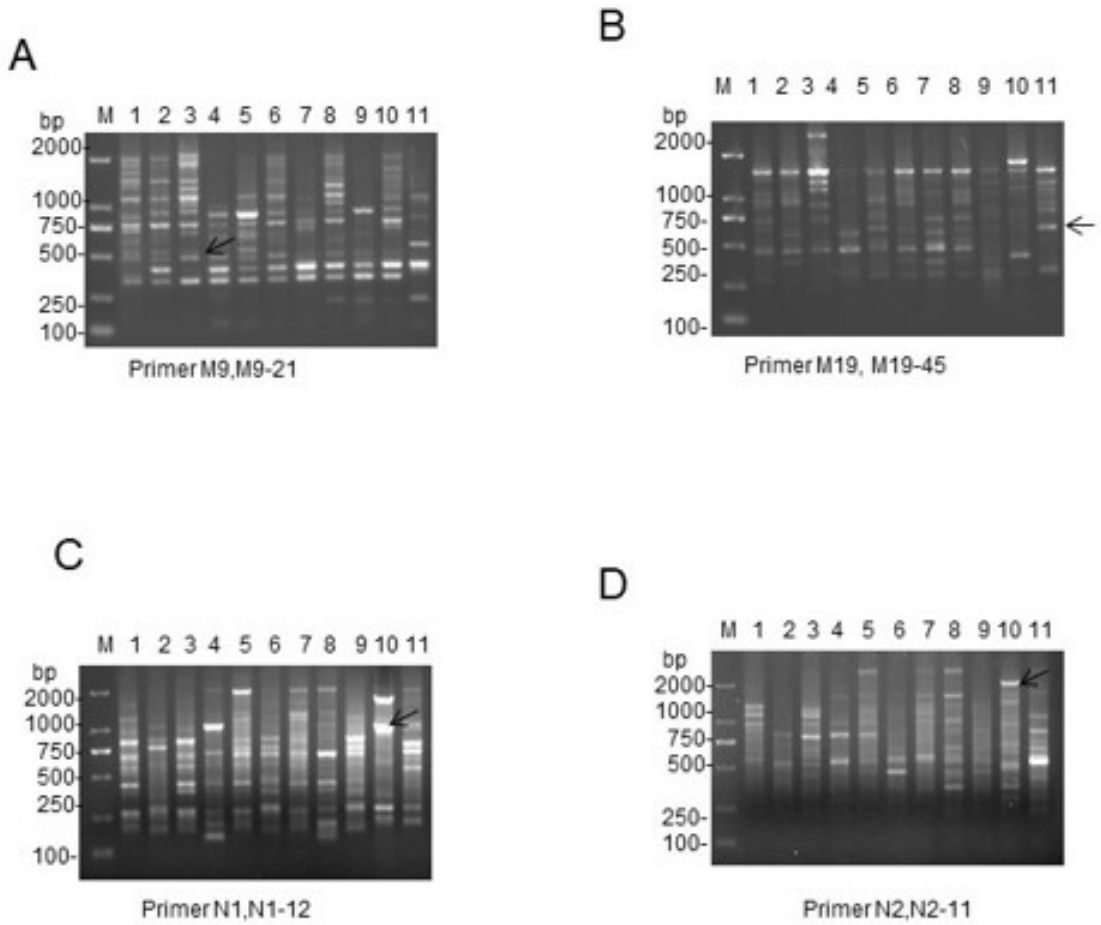

Figure 2. Improved RAPD amplification from DNA samples of *D.nobile* (listed in Table 1) using different RAPD primers. A. primers M9, M9-21; B. primers M19, M19-45; C. primers N1, N1-12; D. primers N2, N2-11.

In LB agar plate, positive clones were screened out (not shown in figure), and those clones were identified by PCR reaction using SP6/T7 primers. The results of the verification of positive clones by PCR are presented in Figure 3. In Figure 3A, the M9-21 positive clone with inserted DNA-fragment with size 514bp, is shown in lane 3, whereas the positive clone M19-45 is shown in Figure 3A as 2 inserted fragments with 795bp in size. In Figure 3B, the positive clone N1-12 is shown in lane 2 (with inserted fragment with size ~1100bp), whereas positive clone N2-11, shown in Figure 3B,has 3 inserted DNA-fragments with ~2100bp in size. EcoRI digestion results are shown in Figure 4, indicating the location site at pGEM -T vector is near to ligation ends. The specific fragments inserts were then subjected to DNA sequencing.

3.2. RAPD-fragments sequencing and characterization

M9-21, M19-45, N1-12, N2-11 RAPD fragment clones were sequenced, and BLAST search was performed for the nucleotide sequences in GenBank database, which indicated that four clones had no significant similarity with any genomic sequence from other species in database. Clone M9-21 (514 nucleotides) was then deposited into GenBank with accession number MZ417502 (Figure 5A); clone M19-45 (795 nucleotides) was deposited into GenBank with accession number MZ484089 (Figure 5B);

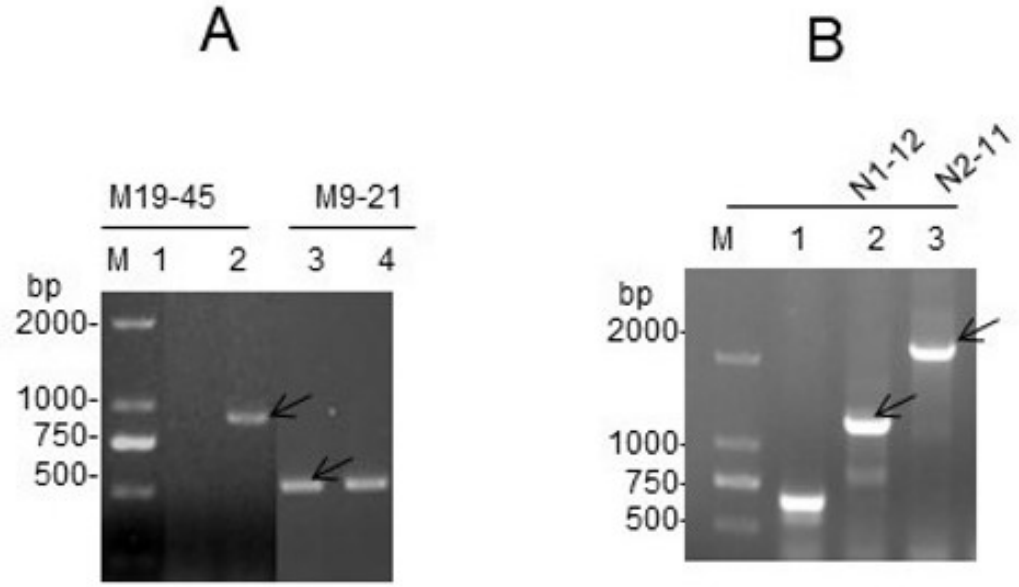

Figure 3. Identification of positive clone M19-45, M9-21 (A), and N1-12, N2-11 (B) by PCR amplification with vector T7/sp6 primers.

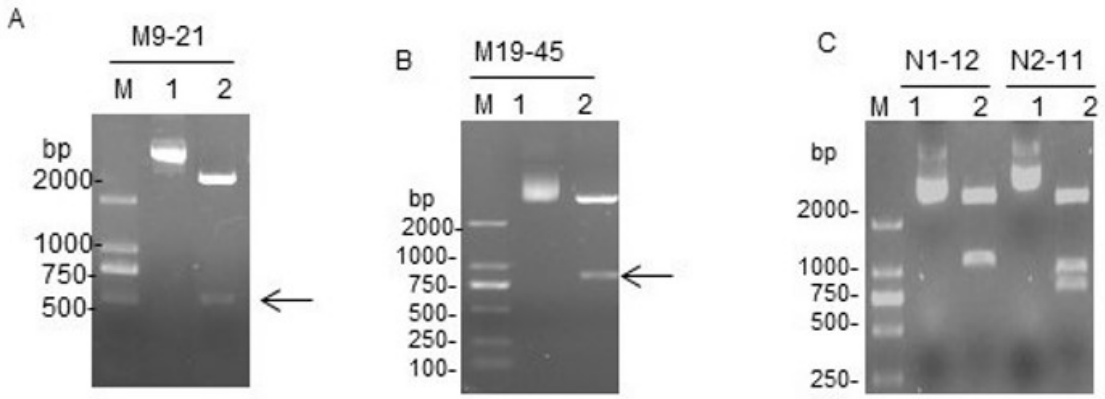

Figure 4. Clone identification of clones M9-21 (A), M19-45 (B), N1-12 & N2-11 (C) by without (lanes 1) or with (lanes 2) EcoRI digestion. The black arrows represent desired PCR product or specific insert bands in different clones. Lane M indicates the DNA molecular weight marker DL2000 with the fragment size (bp).

clone N1-12 (761 nucleotides) was deposited into GenBank with accession number MZ417504 (Figure 5C); and clone N2-11 (718 nucleotides) was deposited into GenBank with accession number MZ417505 (Figure 5D).

3.3. Authentication of Dendrobium nobile Lindl with specific SCAR markers

Four pairs of primers were designed and synthesized based on the cloned sequence of this study, M9-21, M19-45, N1-12, N2-11 to generate stable specific SCAR markers for *D. nobile*. Primer lists are given in Table 2. Genomic DNA from 21 samples used in this study was then amplified using these SCAR primer pairs. The PCR amplification results indicated that four SCAR markers M19-45, M9-21, N1-12 and N2-11 are specific to *Dendrobium officinale Kimura* et Migo (Figure 6A), *D. nobile* in Hejiang of Luzhou (Figure 6B), *D. nobile* in The Republic of the Union of Myanmar (Figure 6C-6D). For other cultivars or species, no amplification was found. Therefore, we report that SCAR markers were developed successfully to identify and authenticate *D. nobile*, and these can be used for genetic characterization of *D. nobile*.

4. Discussion

Genetic characterization or identification is very important ecologically and phylogenetically. With the advancement of molecular biological techniques, this is very easy now-a-days, especially SCAR markers can specifically identify certain species or variants. In recent years, we have developed SCAR markers to identify and characterize several plant species, for example, *Angelica*

A M9-21

GTGATTTGGGTCTCCATCTCTCTTGAATTCGGTCTTTGATTGCAGTTTCCTGTCAGTTAACAGCAATACGCATTGGTTATTATATTACAAATTTCAATATGTTGATAACCAAATCAAATGTTTGGACTGG 130
TCTTTGCTTACCATGATATGACAGCCCTCCTCAAACTTTTCAAGAAAACCTGCAACCCAGCGATCCGCATTCTCCAACCATTCATTGTGAACAATCCCTGCTGTCTTTGCTACCGTATGAAACTAAAGAA 260
TGCAAAGGAAACTGTCATGATTTCCATGAAACTCCATATACATCAAACTGAATTAAGGTAGCATTCTCTTCAAATTATTGGTCATTTCTTCAAATGAAAAGCGGAAAATTGATTTTCCACCATACTTTTA 390
CTCAACTGAACAAGTGGATCTAAAGTAGTATCCATTTAATTTCTTGACCGTTTGCATTTTGATAGGAAAAATTTGAAGATCTCAAAAATTTCCTATCAATTTTCGGAAAACAGCAAAAATTCTC 514

B M19-45

CATCCTAGATCATGTTTGGACTAGTTATATTCACGCAAACAAAATATAATTAGTATAAGGAACTTTCTTTTGGATTACTAGAGCAGCAATAGGTGCCATGGAGAGTCTTGAAAATACAAAGACCCTCTTG 130
GGAGCCTTCTCATCAATAGCTTCAGTAGTAGGCTTTTTAACGACACCTTTGATAGAAGGTACGCTAACAATATTCTCAAAGTCAGCAATTCTCAGTTACTGCTAATTATTTTGTACTAGGATTCTAGAGA 260
GAGAAGCTCTGTGATTCTAGAGAGAGAAAGTGTTTTTTTTATTCTTCATTTTCACGGGCTCCCTGTCTCTAGCCATGGCGGCTGCCGGCCGTCCGCCATCTGATCCTCCTAATCAGCCTCAACTTGTTGG 390
TCCTCAATTAGGGCCTATTCCAATCATTCCACTACCATCCCCTCGATCTTCCATCTTTGGATGCGATGTTAGTTTTGTTTCAAGATCAGGTTCTAAACCTTTGATAATTCAGGAAGGTTTCGGATCTATT 520
GCTCAATATGATAATTCGTCATCCACTATGGATGGAAAAGGTAAGGCTAAGATGGGTTCGTCGGTTCTTGATCTTCATGGTCACACCATTCCGCCGATTGTTAATCTCGTTTCTAAGGGATTCACCTTTG 650
GATCTCTAGAACATGATGTATTGGACTCTCCGATTCTTCCTGAATCTGTTGCTGGTTACACTTCTAACTCGATTACTACTGTTCCATTGGATATTAATAAATCTGAGGTTTTTGTCGCGGTCGCAGATAA 780
TCCTAAGAACTCTTT 795

C N1-12

GCTGATGCCACCCACTTTCATATAGATAAAAGGAAACAGGTTGGCCAGCAGCAGCATAAACAGCGCGACCAGCGCGTAAGCCGTGGGACGCTGGCGAGGCGCGTCCCACGTGGTGGTTAACGTTGTACCG 130
CAGCGCGGGCAGGCGGCTTTCTGGCGATGCTCAAGCTGCGGCAAAGCCACCAGCAAATCACATTGGGGACATAAAATATGTCGCGCAGCATGATGGTGATCGCACATGACAACTCCTCGGCTCAGGCGCC 260
GTTCTTTAATCCTTCGAGATATTCCCAGCGTTCAAAGGCGGCTTCCAGCGCCTGCTCCGCTTCGGCCAGCTGCGCCAGCACTTGCTGCGTACGATCGTGCGATTGGCCAAAGAAGTCAGGATCGGCTACC 390
TGCTCCTGCAGCGTTTGCAGTTCGGCCTCAAGCGTTTCCAGCTTCTGCGGCAGTTGCTCCAGCTCGCGCTGCAGGTTATAGCTTAGTTTGTTCCCGCCGCGTTTGACAGTTTCTGCTTTTGGCTGAGCGA 520
CTTCCGTCGTTTTTTTAACGATCGTCTGCTTTTGCGCCAAAGACTGGGCCTGCTGACCGCGGGCGTCGTGATAACCGCCGACGTACTGACCAATATGGCCGCCGCCTTCGAAGATCCAGCACTCGGTCAC 650
GGTATTGTCGACAAACTGACGATCGTGGCTGACCAGCATCACGGTGCCCTGATAGCCATCGATCAGCTCTTCCAGCAGCTCCAGCGTTTCGACATCAAGGTCGTTTGTCGG 761

D N2-11

GTATGACAGCGGTTATCAGTTCGTCAATCGAGTCCAGCCTTGGTCTGACTCAACTGGCGCGTATTGCCGCATGGCTCACCCCTGACACCATCCCGGGTCTCGATACGTTAAACCTGATGCAGGCTCAGCT 130
TGTGCGTCGCTGGCCGGATAGCCCATTACCCTGCCATAGCGTTGAGATGCTGGAACCACTGCTATGACGTTCAATACATGGCCCTGGCATGACTGGCGCGAAATGCGTGGCGATGAGCCCGCAATTCGCT 260
TGCAGGATGAGACTTTAAGCTGGCGCGACCTGTGTGCGCGCGTCGATGCGCTGGCCGCCGGTTTTGCCCGCCAGGGGGTGAGCGAGGGCGATGGTGTGATGCTACGTGGGCGAAACCAGCCTCAGATATT 390
GTTAGCGTGGCTGGCGTTACTGCAATGTGGCGCGCGTGTATTGCCGGTCAATCCCCAATTACCCTCGCTGTTGCTGGAGGCATTGCTGCCAACCCTGACGCTGCGTTTTGCTGTTAATCTGGCTGATAGC 520
AATACCTTTGTCGGTCTGAAGCCTCTTGAACTGCGACCTGTTGCCCAATCTTATGCTATTGACTGGCAGCCACAACGGCTGGCGTCGATGACCCTGACTTCGGGTTCAACAGGTTTACCTAAGGCGGCGG 650
TCCACACCTGCGCGGCACACCTCGCCAGCGCACAAGGGGTACTGGCGATGATTCCTTTCGCGCCAGAA 718

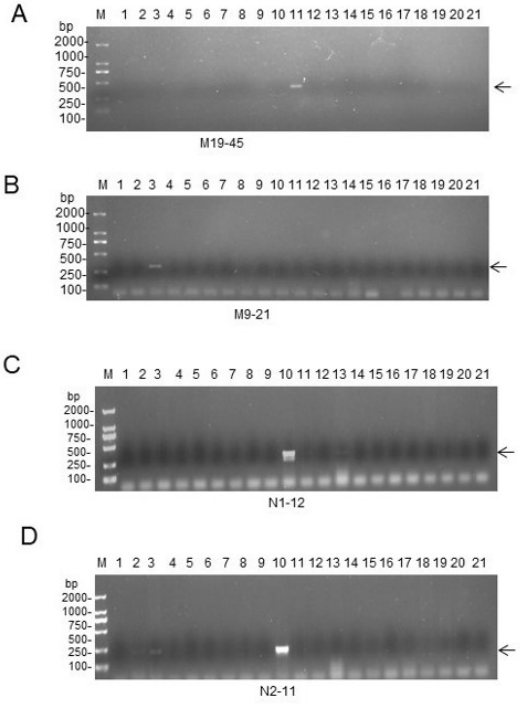

Figure 6. Development of stable RAPD-SCAR markers for M19-45 (A), M9-21 (B), N1-12 (C), N2-11 (D). Lane 1-10 is *D. nobile*, 11-21 is *D. officinale* Kimura et Migo, *Lycium barbarum* L., *Litchi chinensis* Sonn., *Ginkgo biloba* L., *Canarium album* (Lour.) Raeusch., *Lonicera japonica* Thunb., *Mentha haplocalyx* Briq., *Penthorum chinense* Pursh, *Angelica sinensis*, *Gardenia jasminoides*, *Ganoderma lucidum* (Leyss. ex Fr.) Karst.. *Lane M indicates the DNA molecular weight marker DL2000*.

Figure 5. The cloned nucleotide information by Sanger-sequencing. A. The sequences of clone M9-21 with 514bp [The GenBank accession number: MZ417502]. B. The sequences of clone M19-45 with 795bp [The GenBank accession number: MZ484089]. C. The sequences of clone N1-12 with 761bp [The GenBank accession number: MZ417504]. D. The sequences of clone N2-11 with 718bp [The GenBank accession number: MZ417505].

acutiloba, *A.sinensis*, *Levisticumofficinale*, *Lyciumchinense* Miller, *Lonicera japonica*, *Ganoderma lucidium (*Leysser Fr.) Karst etc. (Yang et al., 2014; Zhang et al., 2015; Khan et al., 2016; Liu et al., 2020).

Here in this study, we focused on the development of SCAR marks to genetically identify *D. nobile* from different geographical regions of China. RAPD based studies indicate that the variants of this plant from geographically distant places and from different geological areas possess high level of variations genetically, and interestingly this genetic variations also affects the medicinal properties of this plant (Bhattacharyya et al., 2014; Bhattacharyya and Kumaria, 2015). Here in this study, we found that different *D. nobile* strains from different regions of China are distinct from each other, and based on the differences, we have developed SCAR markers which are specific to the cultivars of *D. nobile* from Hejiang of Luzhou. These markers are deposited in GenBank (accession number: MZ417502, MZ484089, MZ417504, MZ417505). As these markers showed no similarity with other sequences in database, we indicate that these SCAR markers are unique in nature, and they are novel.

The quality of medicinal materials in different regions is different due to different climates and varieties. Authentic medicinal materials are recognized as the best medicinal materials. Therefore, distinguishing authentic medicinal materials is particularly important for medicinal effects. Because *D. nobile* is sold in many regions and even counterfeit products are sold in many areas and on the internet. And the drug effect of *D. nobile* in Luzhou area is the best, so finding a molecular marker that distinguishes this variety appears to be especially important to protect the efficacy of the drug. This experiment has found a molecular

marker that distinguishes this kind of authentic medicinal materials, which provides an important theoretical basis for the protection and production of the drug.

This study, for the first time, developed some novel SCAR biomarkers to authenticate *D. nobile* from a specific region of China that can be further used for the genetic characterization of medicinal plant *D. nobile* from a specific region of China. These SCAR markers could show a more reliable and specific way to identify and characterize *D. nobile* by many morphological methods used traditionally, and thus can play important role in biological conservation of *D. nobile*, and save this plant from extinction in future.

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