Electronic Journal of Biotechnology 18 (2015) 35–39

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Electronic Journal of Biotechnology

Development and significance of RAPD-SCAR markers for the identification of *Litchi chinensis* Sonn. by improved RAPD amplification and molecular cloning

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ARTICLE INFO

Article history:
Received 5 August 2014
Accepted 5 November 2014
Available online 4 December 2014

Keywords:
Genetic authentication
Molecular cloning
Random amplified polymorphic DNA
Sequence-characterized amplified region marker

ABSTRACT

Background: Analysis of genetic diversity is important for the authentication of a species. Litchi (*Litchi chinensis* Sonn.) is a subtropical evergreen tree. Recently, *L. chinensis* has been characterized by an improved random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) analysis. The goal of this study was to develop sequence-characterized amplified region (SCAR) markers from the improved RAPD fragments for the genetic analysis of *L. chinensis*.
Results: The improved RAPD fragments from *L. chinensis* were cloned, sequenced and converted into stable SCAR markers. Sequencing of three cloned RAPD fragments revealed that the clone L7-16 consisted of 222 nucleotides (GenBank accession number KM235222), clone L9-6 consisted of 648 nucleotides (GenBank accession number KM235223), and clone L11-26 consisted of 369 nucleotides (GenBank accession number KM235224). Then, specific primers for SCAR markers L7-16, L9-6, and L11-26 were designed and synthesized. PCR amplification was performed using DNA templates from 24 different samples, including 6 samples of *L. chinensis* and other plants. The SCAR marker L9-6 was specific for all of the *L. chinensis* samples, the SCAR marker L11-26 specific for five *L. chinensis* samples, and the SCAR marker L7-16 only specific for the samples from Luzhou.
Conclusions: This study developed stable SCAR markers for the identification of *L. chinensis* by the cloning of the improved RAPD fragments. Combining RAPD and SCAR markers provides a simple and reliable tool for the genetic characterization of plant species.

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1. Introduction

Analysis of genetic diversity is important for the identification and authentication of a species. This is also important for the genetic profiling and conservation of organisms. For the analysis of genetic diversity, a number of molecular marker techniques have been developed over the last thirty years. These include random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), inter-retrotransposon amplified polymorphism (IRAP), inter-simple sequence repeat (ISSR), internal transcribed spacer (ITS), restriction fragment length polymorphism (RFLP), and amplified fragment length polymorphism (AFLP) analysis. These analyses have all been used for the genetic characterization and authentication of unicellular and multicellular organisms [1,2,3,4].

Litchi (*Litchi chinensis* Sonn., *L. chinensis*), belongs to the family Sapindaceae, and a tropical and subtropical evergreen tree from Hainan, Guangdong, and Fujian in China. It has been cultivated in China since 2000 B.C., and cultivated in many other parts of the world, particularly in Southeast Asia. Litchi is an edible fruit and is used in traditional medicine. As a traditional medicine, the fruit and its secondary metabolic products have been reported to have anticancer, anti-inflammatory, antifungal, antiviral, antioxidant, antiplatelet and anticoagulant, and antidiabetic activities [5,6,7,8,9,10,11,12].

There are numerous litchi cultivars and therefore considerable confusion has arisen regarding their naming and identification. The same cultivar grown in different climates may produce different fruits. Cultivars also have different synonyms in various parts of the world. Therefore, the nutritional or medicinal values of litchi may vary. There are limited studies on genetic diversity of this edible and medicinal species. Recently, we characterized litchi cultivars by employing an improved RAPD and ISSR analysis [13].

Sequence-characterized amplified region (SCAR) markers are stable molecular markers derived from RAPD. The basic principle is to convert the dominant markers into co-dominant markers to reduce the tediousness of RAPD by molecular cloning [14,15,16]. SCAR markers usually have a high level of polymorphism owing to higher annealing temperatures, and longer primers with sequence specificity [17]. When

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2014.11.004
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RAPD is combined with SCAR markers, the procedure becomes a simple PCR analysis using PCR primers designed from the sequence of RAPD amplicons [13,16,17], as indicated in our earlier research [18,19,20,21]. In this study, we used DNA samples of *L. chinensis* collected from Fujian, Hainan, Guangdong, Guangxi and Sichuan, and a DNA sample of *Dimocarpus confinis* from Guangxi to improve RAPD amplification from our previous study [13]. The RAPD bands were cut and purified from agarose gel, followed by DNA ligation and sequencing. Then the SCAR markers were developed for authentication and validation of *L. chinensis*.

2. Materials and methods

2.1. DNA extraction of L. chinensis and other samples

DNA was extracted from fresh tender leaves of different samples of *L. chinensis* (Table 1) and other species by using a previously described slightly modified CTAB method, diluted with 1 × TE buffer to make the final concentration of 10 ng/μL, and stored at -20°C [3,13,18,19].

2.2. Amplification of DNA by improved RAPD

In improved RAPD PCR, the DNA of *L. chinensis* and *D. confinis* was initially amplified using random primers SBC-I10, SBC-A12 and SBC-A16 and Tiangen reagents (Beijing, China). A total of 15 μL PCR reaction system consisted of 7.5 μL 2 × Taq PCR MasterMix, 1.5 μL 2.5 μM primer, 1.5 μL genomic DNA, and ddH_2O. Amplification reactions were performed in an Applied Biosystems Veriti® 96-Well Thermal Cycler (Life Technology, USA) under the following program: initial denaturation at 95°C for 90 s, 40 cycles of denaturation at 94°C for 40 s, annealing at 36°C for 60 s with the RAMP rate from annealing to extension being 0.125°C/s (5% ramp rate), extension at 72°C for 90 s, and a final extension at 72°C for 5 min. PCR products were loaded into a 1.5% agarose gel for electrophoresis.

2.3. Cloning, identification and sequencing of DNA fragments

Three different bright bands were excised from the agarose gel and purified by using TIANgel Mini Purification Kit (DP209, China). Purified DNA fragments were ligated into pGM-T vector (No. VT202) (Tiangen Biotech, Beijing, China) and transformed into *Escherichia coli* (*E. coli*) DH5α competent cells. The recombinant clones were selected on LB agar plates, containing 100 μg/μL of ampicillin, 40 mg of X-gal and 160 μg of IPTG at 37°C overnight. The white colonies were screened out by the blue white screening method. The insertion was verified by PCR by using T7/SP6 primer pairs (T7 primer: 5′-TAATACGACTCACTATAG GG-3′, SP6 primer: 5′-ATTTAGGTGACACTATAGAA-3′), and then by *EcoR* I digestion for a 1% agarose gel electrophoresis [18,19,20]. The cloned DNA fragments were then sequenced using the Sanger method.

2.4. Bioinformatics analysis by online program BLAST

The sequenced DNA was blasted in GenBank database by online on program (http://www.ncbi.nlm.nih.gov/BLAST/) to search for homologous sequences from different species and the homology analyzed.

Table 1
Sources of RAPD-SCAR samples for *L. chinensis* and *D. confinis*.

No.	Species/cultivars	Sources
1	*D. confinis*	Nanning, Guangxi
2	*Heiye, L. chinensis*	Quanzhou, Fujian
3	*Longqiao, L. chinensis*	Wanning, Hainan
4	*Samyuehong, L. chinensis*	Dongguan, Guangdong
5	*Heli, L. chinensis*	Yulin, Guangxi
6	*Dahongpao, L. chinensis*	Zhangba, Luzhou, Sichuan
7	*Dahongpao, L. chinensis*	Hejiang, Luzhou, Sichuan

2.5. Design of SCAR primers

The nucleotide sequence of each of the cloned RAPD fragment was used to design SCAR primers using online Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). The sequences of primers, optimized PCR conditions and amplification lengths are listed in Table 2.

2.6. Development SCAR markers and SCAR analysis

To develop SCAR markers, the PCR amplification was performed using the DNA template from 11 different species, including six cultivars of *L. chinensis* and another 10 medicinal plants (13 samples in total). The 10 μL PCR reaction system was prepared as follows: 5 μL of 2L Taq PCR MasterMix, 1 μL of 2.5 μM SCAR primers, and 1 μL of genomic DNA (10 ng), with the remaining volumes filled by ddH_2O. PCR was performed in an Applied Biosystems Veriti® 96-Well Thermal Cycler (Life Technology, USA) with an initial pre-denaturation for 90 s at 95°C followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 30 s, and extension at 72°C for 40 s. The final extension step was performed at 72°C for 5 min. The amplified PCR products were separated by electrophoresis on a 1.8% agarose gel in 1 × TAE buffer. Gels were visualized by 0.5 μg/mL ethidium bromide staining and the images documented using the ChemiDoc XRS (Bio-Rad, USA).

3. Results

3.1. Cloning of RAPD amplification fragments

Three RAPD primers SBC-I10 (I10), SBC-A12 (A12) and SBC-A16 (A16) were used for improved RAPD amplification in six samples of *L. chinensis* and one sample of *D. confinis* (Table 1) [13]. The results are shown in Fig. 1, where the blue arrows indicate the bands labeled with L7 by primer I10 (Fig. 1a), L9 by primer A12 (Fig. 1b) and L11 by primer A16 (Fig. 1c). The indicated bands were cut from the agarose gel and purified, and ligated to T-vector by AT cloning. The blue and white screening method was adopted firstly to screen the positive clones on LB agar plate (data not shown). The white clones were then identified by PCR amplification using SP6/T7 primer pair (Fig. 2a, b and c). The selected positive clones L7-16, L9-6 and L11-26 were finally identified by extraction of plasmid and DNA digestion by using *EcoR* I enzyme (Fig. 2d). In Fig. 2d, clone L7-16 is shown in lane 2 as an inserted DNA-fragment with ~250 bp in size, clone L9-6 is shown in lane 4 as two inserted DNA-fragments with ~550 bp and ~150 bp in size, and clone L11-26 is shown in lane 6 as an inserted DNA-fragment with ~400 bp in size, respectively. Clones L7-16, L9-6 and L11-26 were finally selected for Sanger sequencing.

3.2. Sequencing and characterization of L. chinensis-specific RAPD fragments

Sequencing of the above mentioned three cloned RAPD fragments of *L. chinensis* revealed that clone L7-16 consisted of 222 nucleotides, which was deposited into GenBank with accession number KM235222 (Fig. 3a); clone L9-6 consisted of 648 nucleotides, which was deposited into GenBank with accession number KM235223 (Fig. 3b); and clone L11-26 consisted of 369 nucleotides, which was deposited into GenBank with accession number KM235224 (Fig. 3c).

BLAST searches of the nucleotide sequences in GenBank database indicated that no clone showed any significant identity to that of any other species (data not shown).

3.3. Development of L. chinensis-specific SCAR markers, and analysis of the PCR amplicons in different species

To generate stable *L. chinensis*-specific diagnostic SCAR markers from RAPD markers, three pairs of primers (L7-16, L9-6 and L11-26)

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