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Full Length Research Paper

A novel polymerase chain reaction (PCR) for rapid isolation of a new *rbcS* gene from *Lemna minor*

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This study developed a novel polymerase chain reaction (PCR) method, ligation-mediated self-formed panhandle PCR, for gene or chromosome walking. It combined the advantages of ligation-mediated PCR in its specificity and of panhandle PCR in its efficiency. Self-formed panhandle PCR was used for a new *rbcS* gene walking to isolate 3' downstream and 5' upstream sequence; 1292 bp DNA *rbcS* gene was obtained via 3' walking of *Lemna minor* gemonic DNA and 5' upstream sequence of the new rbcS gene with a length of 1543 bp was isolated from *L. minor* via self-formed panhandle PCR. A novel *rbcS* gene with the size of 2835 bp, which was confirmed by nested-PCR, was obtained by ligation-mediated self-formed panhandle PCR. Ligation-mediated self-formed panhandle PCR was simple and efficient and should have broad applications in the isolation of unknown sequences in genomes.

Key words: Chromosome walking, *Lemna minor*, polymerase chain reaction (PCR), *rbcS* gene, self-formed panhandle.

INTRODUCTION

Over the past years, several strategies have been developed that aimed at identifying genomic fragments adjacent to known DNA sequences, without going through the process of screening genomic libraries (Wang and Guo, 2010). Polymerase chain reaction (PCR) -based methods have increasingly been applied for gene on chromosome walking. Several PCR methods were available for this purpose: i. Inverse PCR (Uchiyama and Watanabe, 2006; Huang and Chen, 2006; Liu et al., 2004; Keim et al., 2004); ii. Ligation-mediated PCR (LM-PCR) (Tonooka et al., 2008; Villalobos et al., 2006; Ren et al., 2005; Yuanxin et al., 2003; Dai et al., 2000); randomly primed PCR (RP-PCR) (Tanabe et al., 2003).

We report here a simplified and effective PCR method, ligation-mediated self-formed panhandle PCR (SEFP-PCR). SEFP-PCR strategy is based on these principles: i. Restriction sites disperse throughout the genomes of double strand DNA in organisms are natural candidacy for panhandle adaptor pairing; ii. A panhandle adaptor can be a combination of a 3'end pairing with the bases of selected restriction sites in genomic DNA to self-form panhandle, and to limit non-specific amplifications, two-round PCR amplifications were employed in the SEFP-PCR protocol: amplify the target template by using a specific primer and panhandle primer to accumulate the

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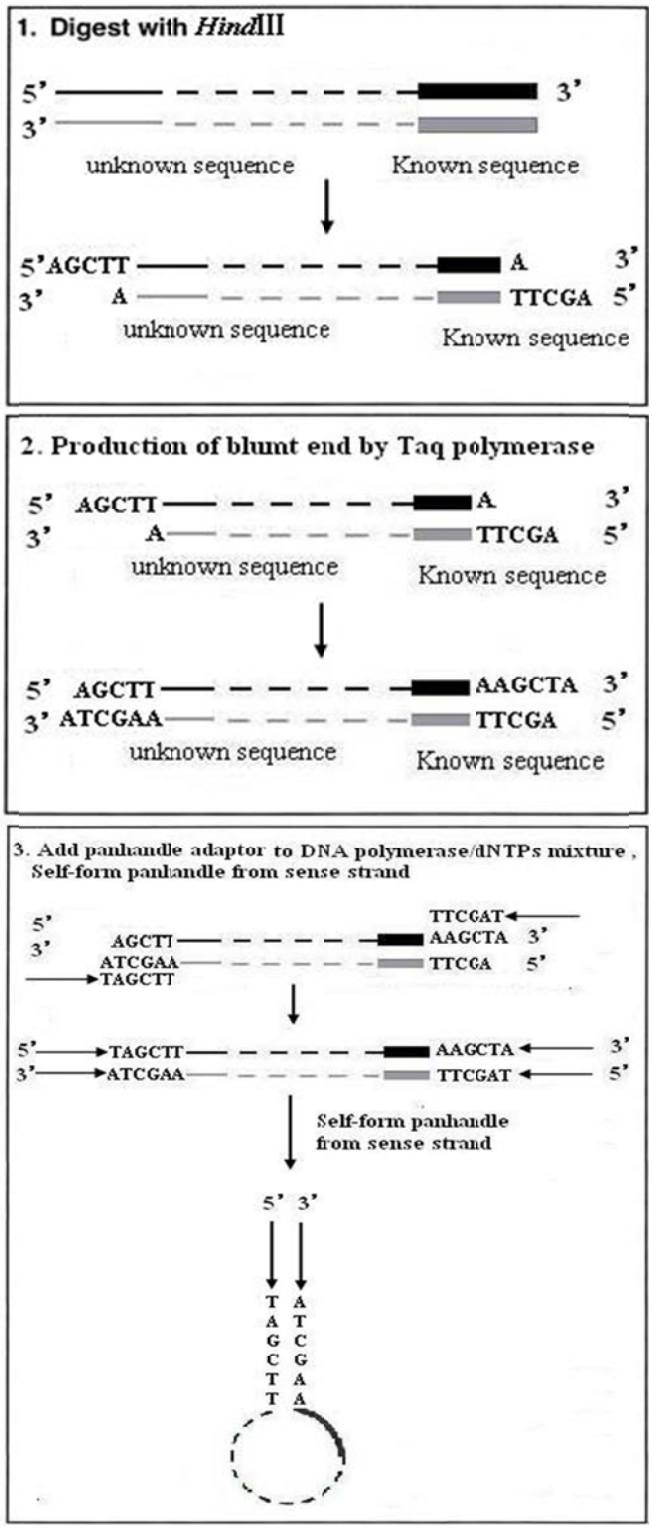

Figure 1. Self-formed panhandle PCR procedures for 5'walking. **Step 1.** *LmrbcS* gene contained *Hind*III site, and *L.minor* genomic DNA was digested with *Hind*III. **Step 2.** production of the sticky blunt end of HindIII by PCR; **Step 3.** Add panhandle adaptor to DNA polymerase/dNTPs mixture and form panhandle template. **Step 4.** primary PCR using primers PAP1 and GSP1; s**tep 5.** isolation of 5'flanking region of *LmrbcS* gene by nested PCR using primers PAP2 and GSP2.

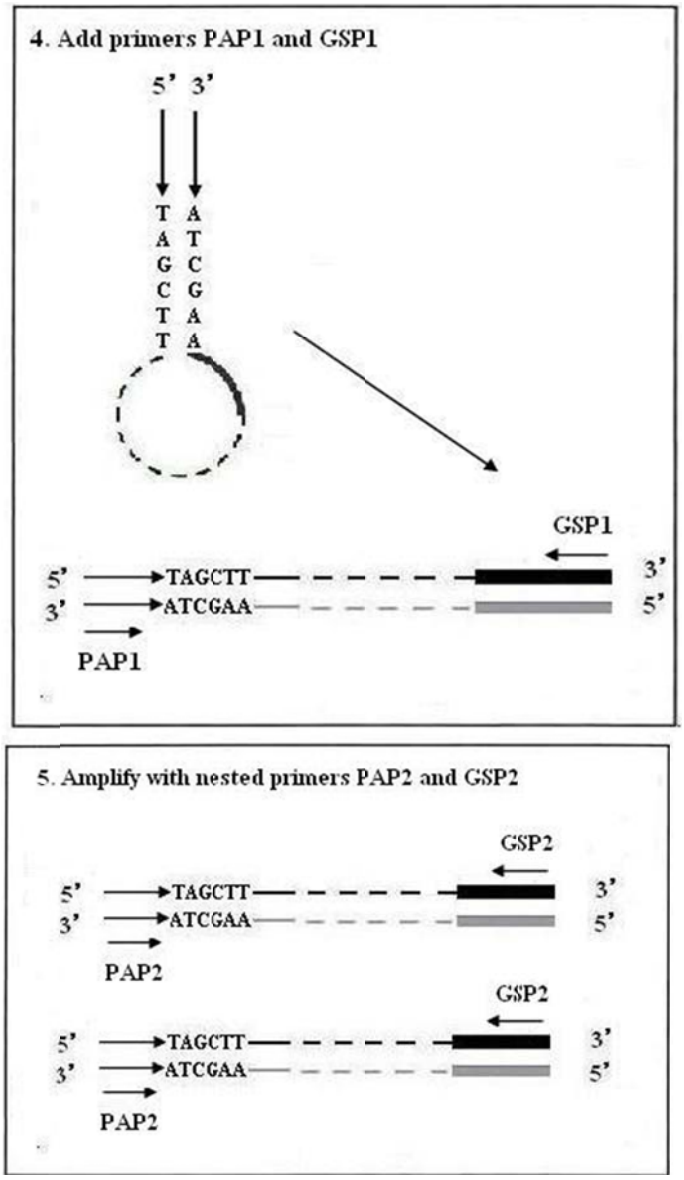

Figure 1. Contd.

specific strand template; and amplify the target gene in the nested-PCR by using the specific primer and panhandle primer primers.

The principle and the procedure of ligation-mediated self-formed panhandle PCR (SEFP-PCR) were outlined in Figure 1, and the detailed thermal cycler settings were listed in Table 1.

MATERIALS AND METHODS

Plant materials and genomic DNA

L. minor was cultured aseptically in SH liquid medium supplemented with 1% sucrose and maintained in a growth chamber at 25°C under cool white fluorescent lighting (90-100 µmol

Table 1. Cycling conditions used for self-formed panhandle PCR

Reaction	Step	Primer	Cycles	Cycling conditions
	2		1	(a) 70°C (3 min);
Self-formed panhandle	3	1 µl of 5 µM panhandle	1	(a) 94°C (3min); (b) 25°C (3 min), then ramping to 70°C at 0.2°C per second; (c) 72°C (4 min);
			1	(a) 94°C (3min), then cool down to 4°C at 0.2°C per second; (b) 4°C (5 min),
Primary	4	3 µl of 5 µM PAP1 and GSP1	20	(a) 94°C (2min); 94°C (1 min) (b) 60-50°C (1 min), touch down (c) 72°C (3 min);
			15	(a) 94°C (2min); 94°C (1 min) (b) 50°C (1 min), (c) 72°C (3 min);
Secondary	5	3 µl of 5 µM PAP2 and GSP2	20	(a) 94°C (2min); 94°C (1 min) (b) 60-50°C (1 min), touch down (c) 72°C (3 min);
			15	(a) 94°C (2min); 94°C (1min) (b) 50°C (1 min), (c) 72°C (3 min);

Table 2. Primers used for cloning of *rbcS* gene from *L.minor*.

Primer	Primers sequence (5' to 3')	Purpose
PF1	MGATAAGRTGTAATCCW	cloning of *rbcS* gene
PR1	TGGAAGCCATCATCGACGAAGCCAT	from *L.minor*
PAP 1	CACGACACGCTACTCAACAC	Adaptor primer
PAP 2	CTCAACACACCACCTCGC	Adaptor primer
3GSP1	GCTTCGTCGATGATGGCTTCCACCG	3'-walking
3 GSP 2	CGGCGACGAGGAAGGCTAACGATCTGT	3'-walking
5 GSP 1	CGGACCTCAGCCCGTTGAAGGGTGC	5'-walking
5 GSP 2	ACGCTGGCCACGGCGGCGGTGGAGC	5'-walking

photons m^{-2} s^{-1}) in a 18/6 h (light/dark) photoperiod. Genomic DNA was isolated from fronds of *L. minor* using the method described by Youru and Sandui (2011).

Isolation of the partial *rbcS* gene from *L. minor*

Based on the known *L. minor rbcS* gene sequences, degeneracy primer pairs (5'- MGATAAGRTGTAATCCW-3') and (5'-TGGAAGCCATCATCGACGAAGCCAT-3') were designed to amplify a new partial *rbcS* gene by PCR. About 400 bp DNA fragment was obtained by PCR.

This DNA fragment was cloned into the pMD18-T vector (TakaRa) for sequencing. Sequence analysis of this DNA fragment revealed that it shared 82-85% identity with the known *L. minor rbcS* gene, which indicated that the 400 bp DNA fragment was partial *rbcS* gene.

Self-formed panhandle PCR

According to the sequence of new *rbcS* gene, primiers were designed for self-formed panhandle PCR (Table 2). The steps of self-formed panhandle PCR (SEFP-PCR) for 5'walking are summarized in Figure 1, and the steps of SEFP-PCR for 3'walking were the same as those for 5'walking except that genomic DNA was digested with *BamH*I; panhandle adaptor sequences and primer sequences are shown in Table 2.

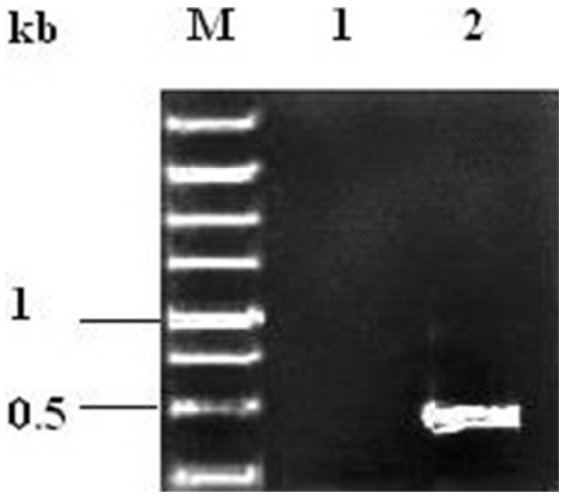

Figure 2. PCR amplification of partial *rbcS* gene from *L. minor.* M, Marker ; 1, negative control; 2, cloning of *LmrbcS* gene from *L. minor.*

***Step 1: Digest genomic DNA with Hind*III**

5 µg genomic DNA was digested with 20 units of *Hind*III (Takara) at 37°C for 2 h. The digested DNA between 2 and 5 kb was purified using DNA extraction kit (*sigma*) and resuspended in ddH_2O for DNA template.

Step 2: Make the sticky end* of *Hind*III *blunt by PCR

The PCR mixture included 4 µL of 10°C long Taq DNA polymerase buffer, 2 µL of mixed dNTP solution (2.5 mM each of dATP, dTTP, dCTP and dGTP), 1.5 U of long Taq DNA polymerase (*Takara*), 20 µL (10-200 ng) template DNA. PCR cycle was run, the detailed thermal cycling conditions for PCR was listed (Table 1).

Step 3: Add panhandle adaptor to DNA polymerase/dNTPs mixture and form panhandle template

Then 1 µL of 5 µM handle adaptor (CACGACACGCTACTCAACACACCACCTCGCACA GCAGTCCNNNNNGGATCC) was added to the PCR mixture , and then PCR cycles were run. The detailed thermal cycling conditions for PCR is listed (Table 1).

Step 4: Add primers PAP1 and GSP1

After heating the reaction mixture at 80°C for 5 min, primers PAP1 and GSP1 were added. Each final 30 µL PCR reaction mixture (30 µL final volume) was the same as that for step 2 except for the template and primers: 0.1 µL of the above PCR product (or diluted 10 times) and 3 µL of 5 µM PAP1 and GSP1 were added to the reaction mixture, then the PCR was run for 35 cycles (Table 1).

Step 5: Add primers PAP2 and GSP2 for nested PCR

Nested PCR was performed after preheating a 29 µL PCR mixture containing all of the reagents except the DNA to 80°C for 5 min to prevent nonspecific priming, and then 1 µL of the diluted PCR products (1000 ×) was added from Step 4 as template. PCR mixture contained 25 µM dNTPs, 5 U of long Taq polymerase, 3 µL of 5µM of gene specific primer (GSP2) and panhandle adaptor primer (PAP2) nested PCR conditions were listed (Table 1).

Cloning and sequencing of PCR products

The nested PCR products were separated on 1.2% agarose gels, then the specific product was purified by PCR purification kit (Sigma) and cloned to T-vector(Takara), and the positive clones were selected for sequencing (Shanghai Baosheng Biotechnology Co. Ltd, China).

Sequence analysis of the 5' flanking region of *LmrbcS* gene

DNA sequence analyses were carried out using the BLAST program (http://ncbi.nlm.nih.gov). The location and distribution of cis-regulatory sequence elements in the *LmrbcS* promoter were analyzed by a signal scan search in the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html). The closest homologues to the *RBCS* promoter were identified by a homology-based search in the PLACE database. The identified *RBCS* homologous fragments were aligned to the *L. minor RBCS* gene promoter using the software program MegAlign and subsequently manually improved.

RESULTS

Cloning of 3'unkonwn region of *LmrbcS* gene from duckweed by SEFP-PCR

Based on the known *rbcS* gene sequence (GenBank accession No. X17231.1, X17230.1, X17232.1, X17235.1, X17234.1 X17233.1, and X00137.1) from *L. minor*, degeneracy primers were designed to obtain a partial *rbcS* gene with a length of 400 bp (Figure 2). Blast analysis showed that this *rbcS* gene (termed *LmrbcS*) shared 80% identity with the known *rbcS* genes from *L. minor.* Another 922 bp DNA fragment was obtained from 3' flanking region of *LmrbcS* via SEFP-PCR (Figure 3). Blast analysis *LmrbcS* gene with the length of 1292 bp was a new *L. minor rbcS* gene since it shared 95% identity with the known *rbcS* genes from *L. minor*.

Identification of 5' flanking region of *LmrbcS* from *L. minor* by SEFP-PCR

In order to allow chromosome walking into the unknown 5'flanking region of *LmrbcS* sequences, according to the DNA sequence of *L.minor LmrbcS* gene, gene-specific primers in nested positions close to the 5' end of the coding regions were designed and synthesized. After two rounds of SEFP-PCR, about 2000 bp fragment was cloned from the 5' upstream region of *LmrbcS* gene by SEFP-PCR (Figure 2). The sequencing results (supplementary material) showed that the cloned product was 1870 bp in length (Figure 4) and DNA sequence analysis (Figure 5) indicated that 1543 bp, which was the

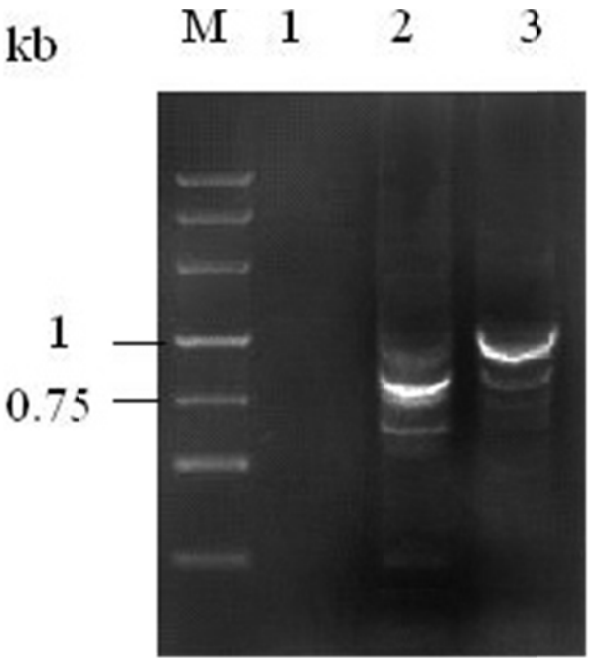

Figure 3. Cloning of 3'flanking region of *LmrbcS* gene from *L. minor* via SEFP-PCR. M, DNA maker; 1, negative control; 2, 2nd round SEFP-PCR; 3, 1st round SEFP-PCR.

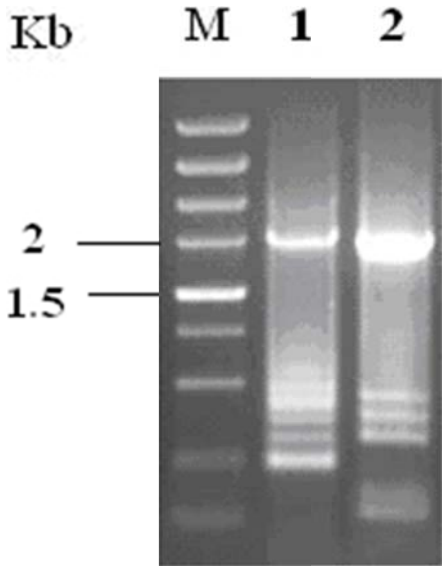

Figure 4. Isolation of 5'flanking region of *LmrbcS* gene from *L. minor* by SEFP-PCR. M, Marker; 1, 1st round I-PCR; 2, nested PCR.

5' flanking region of *LmrbcS*, contained several putative cis-elements, such as sugar responsive elements as well as circadian-box; all present in this *LmrbcS* promoter. The integrity of the genomic DNA (2835 bp in length) was confirmed by nest PCR. Isolated 5' flanking regions were fused to *gus* gene, and tested for expression in tobacco; the isolated 5' flanking regions were shown to drive reporter gene expression in green tissues (data not shown).

TTCAATATCGTAAGCCAGTAATTTTTGACGAATCCCTCTCTCTATATTTGTAGGAAACCCTATTTTTGTCAAATCCCTCACATTTCACA
AATTTGGTATTGATTATTGAATTGGGTACTTCGAATATGTAATTGAGTCAAAATGTCCATCCACGTCCCTTAGGAAGTCAATGCATGGT
ACAACTATTGGGCACCAATTCATTAACAATGCTCAATAGAAAATTCATGCCTAATATTTAACTAGATATCACTTTCTCCCTTCATGGGGT
ATCAGTGTGCGTGTCACCAGGATCAGGGCAAGGATCGAACGACGAACACTAGTTGGAATTTATATAGAGCCTTTGTTTAATGAGGC
ATTTCATGTCTTCTTTGGAACACAAACTAGTTGTCACTTATCTCATATCTTCGTTGGAAAACAAACCAATATCTCACTAATCACGCCGC
TATTGCTTGTGGAAACCAACTAATGACCACTCCTTTAATCCTTAGGCCAAATCAACATTGCCTCCTGGAAGAAGTCACCAAGCCTCT
CCCACTATCAACATTACCCTTGTATGCCATCAAAATTCACAGCAAATTCCACCTTCTCCCACCATGTGTTCTTAGCTACATGAACCTT
AAGTGTCAATTATACTATGCCCCCCTAACAAAACACACCCAAGACAATAATTGGATTTGAATAGGTGAGTTTAATTTATTTTGAAATATT
TAAATCCACCTGACTCCAATAATATCTCACTCTATCACAACAAAGACAAAATCTTCTTATTTTTTTAAGTGTATAATGAAAGTGAAGCAA
GCAAATAAACTTATCCCTAGATAGTAATTCCATACCACTTATCCCATCCCACCTAACACAAGTATCATCCCCTACTCACTCTCCCCTCC
AATAAAACCACTCAAATCCATCAAAACCATACATCTCAAGATAAAGTAATGCAACCCTAAACCACATATCCCTAGGCTTTGTCCTTTTC
CCACAGATAGCCTCTCCATAAAAGTGTAGCCCCTTGAGATAAGACAGTTGTGGAGGAGCGGGTGGATTGTCCCCCCTTGCAGAAG
ATCGGACCATGGCAGCCATAGAATACCCAAACCCTAAGACAGTGACTGTCCCTTATTTAATCAGACCAAGAGCTCTCTCAGAGACC
CCAGAGCTTCCGAGAAGAAGAGGAAGAGAGAGAGAAGGAGAGTGACC**ATG**GCTGCCTCCATGATGAGCTCCACCGCCGCCGTG
GCCAGCGTTGCCAAGACCAGCATGGTCGCACCCTTCAACGGGCTGAGGTCCGCCGTCGCCTTCCCGGCGACCAGGAAGGCCAA
CGATCTGTCGACTCTGCCCAGCAATGGCGGGAGGGTGAGCTGCATGCAGGTGTGGCCGCCGGAGGGGCTGAAGAAGTTCGAGA
CCCTCTCCTACCTCCCTCCCCTCTCCGTCGAGGCTCTCTCCAAGGAGGTCGACTACCTCCTCCGCAACGGCTGGATTCCCTGCG
TTGAGTTCTCCAAGGTATAACAAAACCCATTCTTATCGGTTATCGGATCCTTATCGAATATCATACCCTTTTTGGAATTCTCCAAGGCG
AAAATCTTTCCGATTTTGGTCATCCTTATTGGTACCATTTTCGATAACAATTGGGCCCCGTTTCAGTTAATGGGTCCCATTTTGATACC
GAATCCGTATTGATATCTCCTTCAGTACCGATTGTGTTGTCGAAACGAGTGTTTCTATACTTGATTGATCAGACCCATGTTTTGGTTG
CTAAAATTGATTGGTATCGGATGCCGAAACGGAAACTTTTGATTTTGCGGTTCTTATCGGTACCATTTTCGACCAAAAGTTTCCGTTA
ACGGGTCCCGCTTTGATACCACTATCCGTTATCGGTTACACGATCCAATTCTGATATTGGACCTGTACCAATATCTCTTCTAGTAACG
ATGGCGATATAGATTGCTATCCAATTGCGATATCCATATCAAAATGACTAGTTCGATAGTCGATATCCAGACCCATGTTTGGTATCCAA
TATTGATTGATATTGAATACCAAAAGAGAAAGTATCAGTATTCAATTCTTATCGGTATTGCTACTAGATGCGATGTCGGTACCTGGATG
GGCGAACCCGCAAAGGATTATGAAGGGAAGTGGTGCCGTATCCTTATCGATACCGGTTCAATACCGGTATCCAATACCGATAACAG
CTCCACTATCCAAAGTCAATCGAAGCCGCAAAACCCAAGTGTGTATTAGTATCTAAAATCCGATATTCGTTAACAAAAGGGTTCGTGT
ACCGCCAATACCACGCCTCCCCCGGGTACTACGATGGGCGCTACTGGACCATGTGGAAGCTGCCCATGTTCGGGTGCACCGACG
CCAGCCAGGTGATCGCCGAGGTGGAGGAGGCCAAGAAGGCCTACCCCGAGTATTTCGTCAGAATCATCGGCTTCGACAACAAGC
GCCAAGTCCAGTGCATCAGCTTCATCGCCTACAAGCCCACCTAA

Figure 5. The isolated rbcS gene from *Lemna minor* by SEFP PCR.

DISCUSSION

We have shown that the self-formed panhandle PCR (SEFP-PCR) is an effective method for DNA walking to an unknown genomic region from a known sequence. SEFP-PCR was successful to isolate 1292 bp *rbcs* gene and its 5'upstream sequence of 1543 bp from *L. minor*.

SEFP-PCR combined the advantage of ligation-mediated PCR in its specificity and the advantage of panhandle PCR in its efficiency. Compared with the other existing PCR methods for walking, Self-formed pan-handle PCR has many advantages: (i) High specificity; (ii) a high positive rate of a specific band; iii) Long length of a walking step, theoretically, 4^6 bp (4096 bp) fragment can be obtained by SEFP PCR; and (iv) a high success rate to walk down in one direction.

The advantages of this method are the easy implementation of the procedure, the use of common materials, the relatively few steps needed to amplify the target region, and the high level of specificity achieved in target sequence amplification.

The following aspects of Self-formed panhandle PCR that are different from normal panhandle PCR should be noted: (i) The DNA template concentration should be high to facilitate the creation of the panhandle adaptors; and (ii) Panhandle adaptor should be added at annealing temperature to improve its specificity.

For the general application of this method, it should be noted that the length and efficiency of walking to an unknown region depend on the restriction enzyme used and the frequency of restriction sites in the genomic DNA of the target organism.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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