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ITS and trnL-F Sequences Analysis of *Pulsatilla Chinensis (Bge.) Regel*

Shao-xuan ZHANG [1,a], Guang-zhu LIN [2,b,*], Tian-tian WANG[1,c] and Ya-rong LI[3,d]

[1]Laboratory of Molecular Genetics, Institute of Frontier Medical Sciences, Jilin University, Changchun, China

[2]Cardiolvascular Disease Diagnosis and Treatment Center, The First Bethune Hospital, Jilin University, Changchun, China

[3]Department of Oncology and Hematology, The Second Bethune Hospital, Jilin University, Changchun, China

[a]zhangshaoxuan@jlu.edu.cn, [b]lingz6403@163.com, [c]ttw14@mail.jlu.edu.cn, [d]doctor.lyr@163.com

*Corresponding author

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Abstract. To provide scientific data of the internal transcribed spacer (ITS) and trnL-F sequences for the authentication of *Pulsatilla chinesis(Bge.) Regel.*, we extracted the genome DNA from the leaves of *Pulsatilla chinesis(Bge.) Regel.*, collected in Jilin Province, amplified the ITS and trnL-F region using ITS universal and trnL-F primers of angiosperm, and sequenced the purified PCR products directly. The obtained sequences were edited by Genetyx and reported here.

Introduction

Chinese Anemone, the dried herb of *Pulsatilla chinesis(Bge.) Regel.*(Ranunculaceae), has the functions of detoxicating, removing heat from the blood and relieving diarrhea. It has been prescribed in every edition of ***Chinese Pharmacopoeia*** [1].Although only *Pulsatilla chinesis(Bge.)Regel.* is precribed in ***Chinese Pharmacopoeia***, due to the historical reasons, there are still many area in China use different species of *Pulsatilla* such as *Pulsatilla koreana Nakai.*, *Pulsatilla.dahurica (Fisch.) Spr.*, (distributed in northeast of China); *Pulsatilla turiczaninovii Krylov et Serg.*, *Pulsatilla ambigua Turez ex Pritz.* (distributed in Inner Mongolia, Xinjiang of China) as Chinese Anemone [2].These lead to mistakes in collecting and application, and affect the therapeutic effect of Chinese Anemone. So authentication of *Pulsatilla chinesis(Bge.) Regel.* from the others is necessary for correct use of this drug. Although methods based on traditional appearance, microscopy and thin-layer chromatography have been established and even improved, but due to the similar morphology and chemical composition, it is very hard to authenticate Chinese Anemone from the others used as it by them. Recently, as a result of progresses in molecular biology, molecular authentication has become a new and very reliable method. An accurate method of molecular authentication is generally established based on the data of DNA sequences, such as ITS and chloroplast genes like trnL-F, so sequencing of these regions of *Pulsatilla chinesis(Bge.) Regel.* and the other plants that are used as Chinese Anemone becomes the most foundamental step. In this study, we sequenced the ITS and trnL-F region of *Pulsatilla chinesis (Bge.) Regel.* The obtained sequences were edited and reported here.

Materials and Methods

Materials

Pulsatilla chinesis (Bge.) Regel.. was collected in Jilin Province, China. The sample was authenticated by Professor Wenchang Guo of Jilin University (Table 1). The leaves dried by silica gel were used for DNA extraction.

DNA Extraction

0.3 g leaf was taken from every sample, and put into a culture dish. The leaves were cleaned with cotton swabs to wipe out the impurities with 70% of alcohol. The dried leaves were then grinded to powders in liquid nitrogen and used to extract genome DNA using Plant DNA Isolation Reagent (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China) following the manufacturer's manual. The extracted genome DNA was dissolved in adequate volume of ultrapure water. The quality of the DNA was checked by 1% agarose gel electrophoresis with 5μL of each solution.

Primer Design

The universal primers of ITS [3] and trnL-F [4] were selected and synthesized by Takara Biotechnology (Dalian) Co., Ltd., (Dalian China). The sequences are as follows:

PCR primers:
For ITS:
ITS-F: 5′-TCC ACT GAA CCT TAT CAT TTA G-3′
ITS-R: 5′-CCA TGC TTA AAC TCA GCG GGT-3′
For trnL-F:
trnLF-cF: 5′-CGAAATCGGTAGACGCTACG-3′
trnLK-fR: 5′-ATI'TGAACTGGTGACACGAG-3′
Sequencing primers:
For ITS:
ITS-F:5′-TCC ACT GAA CCT TAT CAT TTA G-3′
In-ITS-3′R:5′-GAC TCG ATG GTT CAC GGG ATT CT-3′
In-ITS-5′F:5′-TCT CGC ATC GAT GAA GAA CG-3′
ITS-R:5′-CCA TGC TTA AAC TCA GCG GGT-3′
For trnL-F:
trnLF-cF:5′-CGAAATCGGTAGACGCTACG-3′
trnLF-dR:5′-GGGGATAGAGGGACTTGAAC-3′
trnLK-eF:5′-GGTTCAAGTCCCTCTATCCC-3′
trnLK-fR:5′-ATI'TGAACTGGTGACACGAG-3′

PCR Amplification

PCR was performed in a 50μL reaction mixture containing 1μL of each 3 PCR primer, 5μL of template DNA, 5μL of 10×Buffer, 5μL of dNTPs, 1μL of Taq DNA polymerase (Takara Biotechnology (Dalian) Co., Ltd., Dalian China). PCR was performed in the following conditions: 1 cylce of 94 ^{o}C for 5 min, 35 cycles of denature at 94 ^{o}C for 1 min, annealing at 55 ^{o}C for 2 min, extension at 72 ^{o}C for 2 min, 1 cycle of 72 ^{o}C for 10 min. All the reactions were performed using MiniCycler PTC-150(MJ Research Inc, St. Bruno, Canada). All the PCR products were checked using a 1% agarose gel.

Sequencing

4 PCR products were purified using Montage PCR Filter Units (Millipore Corporation, USA.) and then sequenced. Sequencing reaction was performed in a 10μL reaction mixture by using ABI BigDye Terminator v3.1 Cycle Sequencing Kit(Applied Biosystems, USA.) with each of the sequencing primers. Sequencing reaction was performed in the following conditions: 1 cycle of 96 ^{o}C for 1 min, 25 cycles of denature at 96 ^{o}C for 30 sec, extension 50 ^{o}C for 5 sec. 1 cycle of 60 ^{o}C for 4 min. After purification, aequences were analyzed by using 3130 sequencer (Applied Biosystems, USA.).

Sequence Analysis

Genetyx-SV/RC version 11.0 (Software Development Co., Ltd., Tokyo, Japan) were used for editing of the sequences.

Results

The information of the purchased plants is shown in Table 1. Three individuals of each sample were used for sequence analysis.

Table 1. the location and date of sample collection

Species	Location	Date of collection
Pulsatilla chinensis (Bge.) Regel.	4 KM from Tuding Town at No.027 Country Road	2013-6-2

The ITS sequences of *Pulsatilla chinensis (Bge.) Regel.*are as follows:

TCGATGCCTGCTCAGCAGAACGACCCGCGAACAAGTGAAAACACCAACTC
ACGCCGGGGAACAGGACGCCGGACAGCCTCACCGCTGCCCCCGACCCGCA
CCCCAGCATAACACAAAAAATCCGGCGCAATTGGCGCCAAGGAATACTTA
CCGGAAATAACGGGTCGACAAGTCGACGCCGTGGATCCGAATACTCAAAC
GACTCTCGGCAACGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGCG
AAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTT
GAACGCAAGTTGCGCCCGAAACCTTTCTGGTCGAGGGCACGTCTGCCTGG
GCGTCACACACAGCGTCGCCCCCACCAAAGCATTTGGATGGGGGCGGAAA
TTGGCCCCCCGAGCCCCCTGGGCACGGTCGGCACAAATGTTGGCCCTCGG
CGGCGAGCGTCGCGGTCAGCGGTGGTTGTACTCTCATCCTCCAAAGACAA
AATGACGCGTCCGCCTCGTCGCCCACTGGGCGAAGATGACCCAAGGAGTC
TCCCCAACCGGAGACTTCCACCT

The trnL-F sequences of *Pulsatilla chinensis (Bge.) Regel.* are as follows:

TACGGACTTGGTTGGATTGAGCCTTGGTATGGAAACATACTAAGTGATAA
CTTTCAAATTCAGAGAAACCCCGGAATTAAAAATGGGCAATCCTGAGCCA
AATCCTTATTTCAGAAAACAAAAGAGGGTTCAGAAAGCAAGAATAAAAA
AAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATAGAGTTGA
CTGCGTTGATCAAGGATTCCTTTTAAACTACAGAAAGGCTGAACCCGTAT
ACATATAATACAAAAATATCAAAGATTAAAAGATTAATGGTCATCCAAAT
CTTTATTTTTTCTTATATGATATACAAAATGGAAGAATTCTTGTGATGTGA
ATCAATTCCAAGTTTAAGTAAGAATCGAATATTCACTGATCAAATAAGTC
ACTCTATAATCTGATAGTTCTTTTAAAGAACTAATTGGACGAGAATAAAG
ATAGAGTCCCATTATACATGTCAATCCCAATACTGACAAAAATGAAATTT
ATAGTAAGAGGAAAATCCGTCGACTTTTGAAATCGTGAGGGTTCAAGTCC
CTCTATCCCCATCCCCAATAAAAAGTTTGCTGTACCCCCTAACTATTTAGT

TTTAGTTAATGTTAATTAATTAAAAGTCATTCTTTTTTTTTTTTAGATTAGT
TATGTTTCTCAGCCATTCTACTCTTTCACAAACGGATCGTAGGAGAAAARG
CCTCTCTTATCACAAGACTTGTGATATATTGTGATATATACAATATATATG
TGATATGTGTAAATCAACATCTAGTAGAAAGGAATTCCCATTTGAATCATT
CACGTTTAATATAATTATTTAAACTTACAAACAAAGTATTCTTTTTGTTTG
AAGATCCAAGACCAAGAAATTACAGGGTTTGGGTAAGACTTTGTAATGCT
TTTTAGTCTATTTAATTGATATACCCAACAAGTACTCGTTTAGAG

Conclusion

The ITS and trnL-F sequences of *Pulsatilla chinensis (Bge.) Regel.*collected in China is for the first time reported.

This study provided scientific data for molecular authentication of *Pulsatilla chinesis(Bge.) Regel.*

Pulsatilla chinesis(Bge.) Regel. distributed in wide area of China, there should be differences among the samples collected in different area, so this study is just a beginning. For authenticating *Pulsatilla chinesis(Bge.) Regel.* more precisely, enlargement of sample from various area and accumulation of these data should be necessary in the future.

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