DNA Extraction and Optimization of ISSR-PCR Reaction System for *Pyracantha*

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Abstract. Three different DNA extraction methods (CTAB, improved CTAB, and nuclear DNA method) were compared in order to isolate high-quality genomic DNA from fresh leaves of *Pyracantha*. The concentration of Mg²⁺, Taq polymerase, dNTPs, primer, and template DNA, greatly influencing ISSR-PCR of *Pyracantha*, were optimized by orthogonal design in this study, and the annealing temperature was also improved. The results showed that the high-quality genomic DNA was obtained by the improved CTAB method and was suitable for ISSR study. The optimal PCR system for ISSR analysis was as follows: total volume 25 µL, 2.5 µL 10×buffer, 1.0 mmol·L⁻¹ Mg²⁺, 0.15 mmol·L⁻¹ dNTPs, 0.1 umol·L⁻¹ primer, 1.2 U Taq DNA polymerase, and 80 ng template DNA. The reaction procedure was as follows: pre-degeneration at 94 °C for 5 min, degeneration at 94 °C for 1 min, annealing at 51.0 °C~59.2 °C for 1 min (annealing temperature depend on different primer), extension at 72 °C for 1 min, 40 cycles, final extension at 72 °C for 5 min and preservation at 4 °C.

1. Introduction

Belonged to the family Rosaceae in the plant kingdom, *Pyracantha* Roem. plants, consisted of about 10 species, are either evergreen thorny shrubs or small trees that are native to Asia and Europe. China is abound of *Pyracantha* resources, as much as 70% of the whole world. Sichuan, Guizhou, Shanxi, and Huibei provence are the distribution center which are famous not only for the amount but also for the largest distribution area[1]. Due to their evergreen leaves, attractive flowers and magnificent red, yellow or orange berries in autumn and winter, *Pyracantha* plants are commonly planted as ornamentals in parks and gardens around the world[2-4]. Also, they are good soil stabilizer for their broad adaptation and capability of wind protection and sand fixation[5-6]. The *Pyracantha* berries could be employed as biosorbent material for the removal of a cationic dye[3, 7].

Over the past 20 years, there have been certain successes in the studies such as biological, morphological introduction and cultivation, propagation, chemical regulation research[2, 4, 5, 8-10]. But there is limited information in the literature about molecular biological research on *Pyracantha*. Inter-simple sequence repeat (ISSR) markers created by Zietkiewicz et al. [11] designs single primer according to microsatellite sequence which widely exist in genome and amplify DNA sequence which has reversely ranged SSR on two sides, besides, its primers have 16 to 18 base. ISSR markers are useful for fingerprinting, mapping, and taxonomic and phylogenetic comparisons, and also provide an alternative strategy for developing microsatellite loci[12-13]. Amplification with ISSR primers requires no prior knowledge of the sequence and are more reproducible and polymorphism than RAPDs[14-15]. ISSR molecular markers has been used in population genetic studies and in detecting clonal diversity[16]. ISSR has also been used for cultivar identification in numerous plant species[17].
Preparation of long, pure DNA has become a major concern for molecular study. Up to now, Cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), high salt but low pH method, and benzyl chloride method are the main methods for DNA extraction[18]. Large number of polysaccharides, flavonoids, triperpenoids, and polyphenols in *Pyracantha* leaves make it difficult to get high quality DNA. Together with some methods for DNA extraction from polyphenols and polysaccharides-rich plants, we have successfully extracted and purified DNA by using Classic CTAB, improved CTAB and nuclear DNA extraction methods on the base of which we have defined the reaction conditions for ISSR-PCR.

2. Materials and methods

2.1. Materials

Details of *Pyracantha* plants were used in the present study are given in table 1. DNA was extracted from young leaves. Unfolded young leaves of *Pyracantha* plants were harvested, washed by clean water and dried by filter paper. After adding 0.1g Vc and PVP respectively, the sample was ground into fine powder in liquid nitrogen. Transfer the mixture to a 1.5 ml centrifuge tube and stored at -70℃ until needed. Total DNA extraction methods were established and optimized by *P. fortuneana* (code: 18), collected in Yucheng district, Sichuan province.

| Code | Species       | Sampling place | Code | Species/Cultivar       | Sampling place |
|------|---------------|----------------|------|------------------------|----------------|
| 1    | *P. fortuneana* | Shijing county, G | 14   | *p. crenulata*         | Shijing county, G |
| 2    | *P. fortuneana* | Shijing county, G | 15   | *p. crenulata* var. kanzuensis | Shijing county, G |
| 3    | *P. fortuneana* | Shijing county, G | 16   | *p. crenulata* var. kanzuensis | Shijing county, G |
| 4    | *P. fortuneana* | Shijing county, G | 17   | *P. fortuneana*        | Yucheng district, S |
| 5    | *P. fortuneana* | Shijing county, G | 18   | *P. fortuneana*        | Yucheng district, S |
| 6    | *P. fortuneana* | Shijing county, G | 19   | *P. fortuneana*        | Yucheng district, S |
| 7    | *P. fortuneana* | Shijing county, G | 20   | *P. fortuneana*        | Yucheng district, S |
| 8    | *P. fortuneana* | Shijing county, G | 21   | *P. fortuneana*        | Yucheng district, S |
| 9    | *P. fortuneana* | Shijing county, G | 22   | *P. fortuneana*        | Yucheng district, S |
| 10   | *P. fortuneana* | Shijing county, G | 23   | *P. fortuneana*        | Yucheng district, S |
| 11   | *P. fortuneana* | Shijing county, G | 24   | *P. fortuneana*        | Hanyuan county, S |
| 12   | *P. crenulata* | Shijing county, G | 25   | *P. fortuneana*        | Hanyuan county, S |
| 13   | *P. crenulata* | Shijing county, G | 26   | *P. fortuneana*        | Hanyuan county, S |

Note: “G” = Guizhou province, “S” = Sichuan province

2.2. Classic CTAB and improved CTAB methods

STEⅠ: 100 mmol·L⁻¹ Tris-HCl (pH 8.0), 50 mmol·L⁻¹ EDTA (pH 8.0), 700 mmol·L⁻¹ NaCl, 3% β-BME, 2% PVP30. STEⅡ: 10 mmol·L⁻¹ Tris-HCl (pH 8.0), 1 mmol·L⁻¹ EDTA (pH 8.0), 100 mmol·L⁻¹ NaCl, 3% β-BME, 2% PVP30. CTABⅠ: 100 mmol·L⁻¹ Tris-HCl (pH 8.0), 20 mmol·L⁻¹ EDTA (pH 8.0), 1.4 mmol·L⁻¹ NaCl, 2% CTAB, 2% β-BME, 2% PVP30. CTABⅡ: 100 mmol·L⁻¹ Tris-HCl (pH 8.0), 20 mmol·L⁻¹ EDTA (pH 8.0), 1.4 mmol·L⁻¹ NaCl, 3% CTAB, 2% β-BME, 2% PVP30. Referring to DNA extraction protocol for plants containing high polysaccharide, polyphenol, and other secondary metabolites such as hawthorn[19] and walnut[20], we made some adjustments to the classic CTAB methods (Table 2.): add 1.5 ml STE I/II (or without), place at room temperature for 5 to 10 min, during
which invert the tubes several times to mix thoroughly with the leaf slurry, centrifuge at 5000 rpm for 5 min at 20℃. The tubes were removed the upper aqueous phase and then add 750 µL CTAB I/II that was preheated to 65℃. Then the tubes were incubated at 65℃ for 45 min and were spun at 12000 rpm for 10 min in a tabletop centrifuge at room temperature. After centrifugation, the top aqueous phase was transferred to a new 1.5 ml centrifuge tube. The equal volume of phenol-chloroform-isoamyl alcohol (v/v/v = 25/24/1) was add to new 1.5 ml centrifuge tube. The tubes were inverted the tubes gently to form an emulsion, and were spun at 12000 rpm for 10 min again. Transfer the upper aqueous phase gently to a fresh tube, add (or without) 0.2 volume of 5 mmol·L⁻¹ LiCl, 2 volumes of cold (-20℃) 95% ethanol. Centrifuge at 12000 rpm for 10 min after storing at -20℃ for 30 min. The supernatant was poured out and then the pellet was washed with cold (0 to 4℃) 75% ethanol. Completely remove ethanol without drying the DNA by leaving the tubes uncovered at 37℃ for 20 to 30 min. Dissolve DNA with 100 µL deionized water at last.

Table 2. Methods of DNA extraction for *P. fortuneana*.

| Treatments | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|------------|---|---|---|---|---|---|---|---|---|----|----|----|
| STE I      | + | + | + | + | - | - | - | - | - | -  | -  | -  |
| STE II     | - | - | - | - | + | + | + | - | - | -  | -  | -  |
| CTAB I     | + | + | - | - | + | + | - | - | - | +  | -  | -  |
| CTAB II    | - | - | + | + | - | - | + | - | - | +  | -  | +  |
| DNA        | + | - | + | - | + | - | + | + | + | -  | +  | -  |

Note: “+” = yes, “-” = no

2.3. Nuclear DNA method
Ice-cooled solution I: 200 mmol·L⁻¹ Tris-HCl (pH 8.0), 50 mmol·L⁻¹ EDTA (pH 8.0), 0.05 mol·L⁻¹ NaCl. Ice-cooled solution II: 100 mmol·L⁻¹ Tris-HCl (pH 8.0), 4 mmol·L⁻¹ EDTA (pH 8.0), 5 mol·L⁻¹ NaCl. CTAB extracting buffer: 100 mmol·L⁻¹ Tris-HCl(pH 8.0), 20 mmol·L⁻¹ EDTA (pH 8.0), 1.4 mol·L⁻¹ DNA, 2% CTAB, 2% β-BME, 2% PVP30. Add 600 µL ice-cooled solution I/II to the prepared powder and incubate on ice for 10 min, spin at 6000 rpm for 10 min, then remove the top aqueous phase, pour into 750 µl CTAB extracting buffer, mix gently and incubate at 65℃ for 45 min. The following treatments are the same as improved CTAB method.

2.4. DNA analysis
Place the tube on a rocking platform and gently rock the solution for 12 to 24 h until the DNA has completely dissolved. The DNA solution was stored at 4℃ and then measured the concentration by absorbance at 260 nm and 280 nm on Eppendorf Biophotometer. Analyze the quality of the preparation of DNA by electrophoresis through a conventional 0.8% agarose gel in 0.5%×TBE buffer. Bands were visualized by staining with ethidium bromide (EB) illuminated with UV light and photographed.

2.5. Optimization of ISSR-PCR compounds by orthogonal design
The PCR was performed in a total volume of 25 µL, containing template DNA, Mg²⁺, dNTPs, primer concentration, and Taq polymerase as described in table 3. And five factors were optimized with primer S60 by using the genomic DNA of *P. fortuneana* in orthogonal test. After pre-PCR heating at 94℃ for 5 min, a reaction cycle of 94℃ for 1 min, and 72℃ for 1 min was repeated for 40 times, followed by final extension at 72℃ for 5 min.

2.6. Identification of the optimal annealing temperature
Appropriate reaction system was identified based on orthogonal experimental results, nine temperature grades, 50℃, 50.8℃, 52.1℃, 53.7℃, 55.6℃, 57.2℃, 58.3℃, 59.2℃, 59.8℃ was generated.
automatically by PTC-200 (Bio-Rad) based on Tm-55°C±4°C of each primer. The PCR reaction programs works exactly like the orthogonal test, except that annealing temperatures were different.

Table 3. Gradient arrays of 5 factors that affected the ISSR-PCR by orthogonal design.

| No. | Mg²⁺ (mmol·L⁻¹) | Taq polymerase (U) | Primer (µmol·L⁻¹) | Template DNA (ng) | dNTPs (mmol·L⁻¹) |
|-----|----------------|-------------------|-------------------|------------------|-----------------|
| 1   | 1.0            | 0.6               | 0.1               | 20               | 0.10            |
| 2   | 1.0            | 0.9               | 0.2               | 40               | 0.15            |
| 3   | 1.0            | 1.2               | 0.3               | 60               | 0.20            |
| 4   | 1.0            | 1.5               | 0.4               | 80               | 0.25            |
| 5   | 1.0            | 1.8               | 0.5               | 100              | 0.30            |
| 6   | 1.5            | 0.6               | 0.2               | 80               | 0.30            |
| 7   | 1.5            | 0.9               | 0.3               | 100              | 0.10            |
| 8   | 1.5            | 1.2               | 0.4               | 20               | 0.15            |
| 9   | 1.5            | 1.5               | 0.5               | 40               | 0.20            |
| 10  | 1.5            | 1.8               | 0.1               | 60               | 0.25            |
| 11  | 2.0            | 0.6               | 0.3               | 40               | 0.25            |
| 12  | 2.0            | 0.9               | 0.4               | 60               | 0.30            |
| 13  | 2.0            | 1.2               | 0.5               | 80               | 0.10            |
| 14  | 2.0            | 1.5               | 0.1               | 100              | 0.15            |
| 15  | 2.0            | 1.8               | 0.2               | 20               | 0.20            |
| 16  | 2.5            | 0.6               | 0.4               | 100              | 0.20            |
| 17  | 2.5            | 0.9               | 0.5               | 20               | 0.25            |
| 18  | 2.5            | 1.2               | 0.1               | 40               | 0.30            |
| 19  | 2.5            | 1.5               | 0.2               | 60               | 0.10            |
| 20  | 2.5            | 1.8               | 0.3               | 80               | 0.15            |
| 21  | 3.0            | 0.6               | 0.5               | 60               | 0.15            |
| 22  | 3.0            | 0.9               | 0.1               | 80               | 0.20            |
| 23  | 3.0            | 1.2               | 0.2               | 100              | 0.25            |
| 24  | 3.0            | 1.5               | 0.3               | 20               | 0.30            |
| 25  | 3.0            | 1.8               | 0.4               | 40               | 0.10            |

2.7. Identification of PCR products
The amplified products were conducted electrophoretic analysis in 1.4% agarose gel then taken photos and preserved through image acquisition and analysis system after stained by 1.5 µg·mL⁻¹ EB for 15min.

3. Results

3.1. Electrophoretic analysis of P. fortuneana DNA extracted by different methods
Results indicated that the methods 2, 3, 4, 7 and 13 successfully isolated DNA but with different quality. Method 4 obtained the best result, while methods 9 to 12 didn’t even isolated DNA. The
loading wells brightened up in lane 5, 7, 9, 10, 11 and 12 mean they were contaminated by polysaccharide of protein or some other secondary metabolites else (Fig 1.).

![Fig 1. Caption of the Figure 1. Below the figure.](image)

![Fig 2. Electrophoretic analysis by improved CTAB method 4.](image)

Note: Lane 1 to 12 using improved CTAB methods, 13,14 DNA extracted by nuclear DNA methods.

3.2. Electrophoretic analysis of P. fortuneana DNA extracted by different methods

We detected that the improved CTAB 4 method was the best protocol for *P. fortuneana* genome DNA, with which method the DNA bands were clear, high brightness after digested by RNase (Fig 2.). Detected by Eppendorf Biophotometer under 260 nm and 280nm, this procedure yielded 350 to 930µg·mL⁻¹ DNA with A260/A280 range from 1.87 to 2.05.

![Fig.3 The electrophoresis results of RAPD-PCR orthogonal (primer S60).](image)

Table 4. Intuitive analysis of orthogonal design.

| Results | Mg²⁺ | Taq polymerase | Primer | Template DNA | dNTPs |
|---------|------|----------------|--------|--------------|-------|
| K₁      | 104  | 66             | 94.25  | 58.25        | 51    |
| K₂      | 51.75| 64.5           | 73.5   | 65.25        | 60    |
| K₃      | 72.5 | 76.5           | 63     | 60.25        | 66.75 |
| K₄      | 45   | 55.5           | 57.5   | 74.25        | 69.75 |
| K₅      | 53   | 64             | 38.25  | 68.5         | 79    |
| k₁      | 20.8 | 13.2           | 18.85  | 11.65        | 10.2  |
| k₂      | 10.35| 12.9           | 14.7   | 13.05        | 12    |
| k₃      | 14.5 | 15.3           | 12.6   | 12.05        | 13.35 |
| k₄      | 9    | 11.1           | 11.5   | 14.85        | 13.95 |
| k₅      | 10.6 | 12.8           | 7.65   | 13.7         | 15.8  |
| R       | 11.8 | 4.2            | 11.2   | 3.2          | 5.6   |

Note: Ki=Σ N, ki=(Σ N)/n. Ki=the sum of scores of each factor at the same level, ki=average mark for each factor at the same level, N=scores for each factor at the same level, n=total levels for each factor.
Fig 4. Effects of annealing temperature on ISSR amplification (primer UBC808).
Note: The annealing temperature from left to right in order: 50℃, 50.8℃, 52.1℃, 53.7℃, 55.6℃, 57.2℃, 58.3℃, 59.2℃, 59.8℃.

3.3. Data scoring and analysis for orthogonal tests
All distinctive and unambiguous polymorphic bands generated by PCR were scored manually by different individuals (with abundance, clear, and high lightened bands got 25 points, the worst scored 1 in contrast). Take the average mark as the last score. 25 treatments (Fig 3.) got 24, 22.25, 21.25, 18.75, 17.75, 16, 6.25, 7.75, 4.75, 17, 14.25, 16, 11.5, 17.25, 13.5, 9.75, 2.25, 18.5, 4, 10.5, 2, 17.5, 17.5, 10.75 and 5.25 point respectively. Refering to the orthogonal design, the sums of each factor at the same level were marked as Ki, the average score for the same level of each factor was labeled as ki and the range (R) showed the maximum differences for vary level of the same factor. The results were showed in table 4. R reflected components’ affections on PCR results. The wider the range was, the more significant the interaction was. We can get a conclusion that among all components in the PCR system, the concentration of Mg$^{2+}$ was the most important factor, then concentration of oligos (primer), dNTPs, Taq DNA polymerase and template DNA in turn. Considering amplified results and costs, the optimal reaction systems was: Mg$^{2+}$ 1.0 mmol·L$^{-1}$, Taq DNA polymerase 1.2 U, primer 0.1 µmol·L$^{-1}$, dNTPs 0.15 mmol·L$^{-1}$ and template DNA 80 ng (Table 4.).

3.4. Influences of annealing temperature on ISSR amplification
Generally speaking, low annealing temperature can cause fuzzy background and more week bands, high annealing temperature can cause less bands, and also against amplification of large molecular weight DNA. Such as primer UBC808, it produced more weeker bands, which was hard to count as annealing temperature is below 55.6℃ (Fig 4.), Fig 4 also showed that clear background, more amplified bands, bright and clear main band were observed in lane 6 and lane 7. Therefore, annealing temperature of 57.2~58.3℃ was optimum. According to gradient test of annealing temperature in optimum ISSR-PCR reaction system of P. fortuneana, it was found that the optimum annealing temperature of UBC808 was 58.3℃ and the annealing temperature was different for different primers (Table 5.).

| Primers | Sequence (5’-3’) | Annealing temperature (℃) | Tm value (℃) | Primers | Sequence (5’-3’) | Annealing temperature (℃) | Tm value (℃) |
|---------|------------------|---------------------------|--------------|---------|------------------|---------------------------|--------------|
| UBC808  | (AG)$_n$C        | 58.3                      | 52.2         | UBC841  | (GA)$_n$GTC      | 58.0                      | 56.2         |
| UBC811  | (GA)$_n$C        | 57.4                      | 54.6         | UBC842  | (GA)$_n$CTG      | 55.6                      | 54.4         |
| UBC814  | (CT)$_n$A        | 52.8                      | 52.2         | UBC856  | (AC)$_n$GTA      | 58.0                      | 53.9         |
| UBC815  | (CT)$_n$G        | 51.7                      | 54.6         | UBC857  | (AC)$_n$GTC      | 58.0                      | 56.2         |
| UBC820  | (GT)$_n$C        | 59.2                      | 56.2         | UBC864  | (ATG)$_n$        | 57.4                      | 48.2         |
| UBC823  | (TC)$_n$C        | 53.7                      | 54.4         | UBC866  | (CT)$_n$C        | 59.2                      | 56.2         |
UBC834 (AG)₈ GTT 58.0 53.9 UBC873 (GACA)₄ 58.5 51.5
UBC835 (AG)₈ GTC 57.4 56.2 UBC876 (GATA)₃ (GACA)₂ 51.0 46.4
UBC836 (AG)₈ GTA 58.0 54.4 UBC878 (GGAT)₄ 52.1 54.4
UBC840 (GA)₈ GTT 54.3 53.9 UBC881 (GGGTG)₃ 58.0 61.8

Fig 5. DNA fragments of 26 germplasm resources of Pyracantha by ISSR-PCR analyzed in 1.4% agarose gel electrophoresis (primer UBC857, M:100bp DNA ladder).

ISSR-PCR by the deduced suitable PCR conditions with primer S221, 26 samples of Pyracantha plants obtained rich and clear bands (Fig 5.).

4. Conclusion
Good quality deoxyribonucleic acid (DNA) is the pre-requisite for its downstream applications. The presence of high concentrations of polysaccharides, polyphenols, proteins, and other secondary metabolites in Pyracantha leaves poses problem in getting good quality DNA fit for polymerase chain reaction (PCR) applications. The two common used protocols for DNA isolation are CTAB and SDS, using for polysaccharides rich plant, CTAB method takes priority. Base on the classic CTAB method, there have been large amount of reports on modifications for different goals such as wiping out polysaccharides, polyphenols ,and other secondary metabolites[21-24]. We can draw a conclusion through our study that there are several key protocols for isolation high quality DNA from Pyracantha plants:

① Grinding. grind the sample to fine powder in liquid nitrogen as quick as possible. Adding a certain amount of Vc and PVP can effectively inhibit polyphenols oxidizing procedure.

② Centrifuge at a relative slow speed(3000 to 5000 rpm) in order to collect the nucleus before cracking the nuclear membrane. During the processing time, SET buffer serves to maintain the integrity of the nuclear membrane.

③ The Pyracantha plants are rich in polysaccharides, phenols and other compounds which are inhibitors to extract and purify DNA. Adding certain amount of PVP and β -BME can effectively solve this problem.

④ All protocols after cracking the nuclear membrane should be carried out gently to avoid mechanical cleavage to free DNA.

⑤ In order to completely get rid of other factors except DNA from the upper aqueous phase, higher speed for centrifuge was recommended, but after getting the DNA pellet a relative lower speed took priority.

⑥ Adding proper volume of high salt solutions such as 1.4 mmol·L⁻¹ NaCl can reduce contaminations of protein, polysaccharide etc. which might also reduce the quantities of DNA was also recommended in the procedure anyway.

Utilizing orthogonal design and quantitative statistics, we have studied the effects of Mg²⁺ concentration, primers, dNTPs concentration, TaqDNA polymerase and DNA templates on ISSR-PCR and successfully established stable and effective reaction systems. The concentration of Mg²⁺, primers, dNTPmix, TaqDNA polymerase have greater impact on amplification but DNA template. Orthogonal desiging which is informative and easy data analyzing but workload reduction can be used as a powerful tool for academic research.
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