

Optimization of chloroplast microsatellite PCR conditions and primer screening for endangered *Rheum officinale*, *Rheum palmatum*, and *Rheum tanguticum*

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Genet. Mol. Res. 13 (3): 5787-5794 (2014)

Received June 11, 2013

Accepted December 2, 2013

Published July 29, 2014

DOI <http://dx.doi.org/10.4238/2014.July.29.6>

ABSTRACT. Chloroplast microsatellite primers were developed in order to provide more population genetic information of endangered *Rheum officinale*, *R. palmatum*, and *R. tanguticum* for conservation. The dried roots and rhizomes of these plants are important in traditional Chinese medicine. The results showed that the optimum concentrations of Mg²⁺, *Taq* DNA polymerase, dNTPs, template DNA, and primers in a 25- μ L reaction system were 2.0 mM, 1.0 U, 0.10 mM, 20 ng, and 0.8 μ M, respectively. Fourteen of 53 primer combinations were chosen for their high clarity and repetition in three species, and their annealing temperatures ranged from 56 to 58°C. These primers and the optimized polymerase chain reaction system may provide a tool for understanding the demography and genetic variation of these endangered plants.

Key words: Rhubarb; Chloroplast simple sequence repeat; Polymerase chain reaction optimization; *Rheum*

INTRODUCTION

Rheum officinale Baill., *R. palmatum* L., and *R. tanguticum* Maxim. ex Balf. (Polygonaceae) are endemic species of China (Bao and Grabovskaya-Borodina, 2003). Their dried roots and rhizomes are important in traditional Chinese medicine (named rhubarb; Chinese Pharmacopoeia Committee, 2010). Bitter in flavor and cold in property, rhubarb is widely used as a purgative and anti-inflammatory agent (Chinese Pharmacopoeia Committee, 2010). The young stem and petioles of three source plants of rhubarb are also taken as vegetables and used in health foods (Su et al., 1998). Recent studies have revealed that rhubarb has anti-cancer properties and immunity function because of its rhein and polysaccharide content (Huang et al., 2007; Gao et al., 2008). These medicinal properties of rhubarb have attracted great interest in pharmaceutical and genetic variation research.

In our previous study, the distribution regions of the three species were found to be overlapping and vicarious (Wang et al., 2010), and no significant genetic difference was found at the species level by inter-simple sequence repeats (ISSRs) (Wang et al., 2012a,b). Although Chen et al. (2009) used simple sequence repeat (SSR) markers to detect the genetic diversity of rhubarb, only one species, *R. tanguticum*, was employed. The chloroplast chromosome is non-recombinant, uniparentally inherited in angiosperms, and effectively haploid. Chloroplast microsatellites (cpSSRs) are usually mononucleotide repeats that evolve faster than the other gene regions in the chloroplast genome (Jakob et al., 2007) and display high polymorphism. Furthermore, the short length and the limited number of alleles of most cpSSRs also permit the confirmation of allele size by sequencing (Weising and Gardner, 1999). This is more difficult for many nuclear SSRs, where the high heterozygosity and long size make distinct sequences difficult to obtain (Siragusa and Carimi, 2009). Therefore, they would be useful markers to gain more insights in genetic relationship studies of closely related species and populations, phylogeographic studies within a species, and marker-assisted selection (Provan et al., 2001; Cubas et al., 2005; Chen et al., 2007; Siragusa and Carimi, 2009; Melotto-Passarin et al., 2011).

In this study, we aimed to develop an efficient protocol of cpSSR-polymerase chain reaction (PCR) conditions and screen 53 primers and select those with high clarity and repetition for further investigation.

MATERIAL AND METHODS

Plant materials

The materials of *R. officinale* (voucher: Nanchuan, Chongqing, altitude of 1832 m, 29°00.008'N, 107°11.769'E, Xu-mei Wang and Xiao-qi Hou 09072609, SANU), *R. palmatum* (voucher: Dari, Qinghai, altitude of 3947 m, 33°49.151'N, 99°42.669'E, Xiao-qi Hou 10081435, SANU) and *R. tanguticum* (voucher: Maqin, Qinghai, altitude of 3373 m, 34°36.955'N, 100°33.970'E, Xiao-qi Hou 10081332, SANU) were collected in 2009 and 2010. Chongqing and Qinghai are both main distribution regions and cultivated drug-producing areas (Wang et al., 2010). Fresh leaf samples from five wild individuals of each species were collected and preserved in zip-lock bags with silica gel until use.

DNA isolation

The dried leaf tissue of *R. officinale*, *R. palmatum*, and *R. tanguticum* were sampled for DNA isolation by the improved 2X cetyltrimethylammonium bromide procedure (Doyle and Doyle, 1987). The quality and quantity of DNA were determined by an ultraviolet spectrophotometer (ND-2000, NanoDrop, USA). The DNA concentration and purity were also determined by electrophoresis on 1.0% agarose gels based on the intensities of bands when compared with 1 kb plus DNA ladder as a marker. The DNA sample was diluted to the template concentration 20 ng/ μ L and stored at -20°C until use.

Optimization of the cpSSR-PCR system and primer screening

A total of 53 cpSSR primers were synthesized by Beijing Dingguo Biotech. Co. Ltd. (China) according to primer characteristics reported in other species (Vendramin et al., 1996; Weising and Gardner, 1999; Cheng et al., 2003; Chung and Staub, 2003; Sebastiani et al., 2004; Hu et al., 2009).

The PCR was carried out in Applied Biosystems Veriti Thermal Cycler (USA), in a total volume of 25 μ L containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100, 2.0 mM MgCl₂, 0.2 mM dNTPs, 0.4 μ M primer, 1 U *Taq* Plus DNA polymerase (Tiangen, China) and 20 ng template DNA. The thermocycler was programmed for an initial denaturation of 5 min at 94°C; 35 cycles of 30 seconds at 94°C, 30 seconds at different annealing temperature for each primer, and extension at 72°C for 50 seconds; and a final extension at 72°C for 8 min. Reactions without DNA were used as negative controls. Six factors including *Taq* DNA polymerase, dNTP, primer, Mg²⁺, and DNA template concentrations and annealing temperatures were estimated as described in Table 1. Eight microliters PCR product was electrophoresed on 2% (w/v) agarose gel, in 1X Tris, borate, and ethylenediaminetetraacetic acid buffer at 110 V for 1.5 h and stained with ethidium bromide (0.5 μ g/mL). Gels with amplification fragments were visualized and photographed in ultraviolet light by a Bio-Rad Gel Documentation System (Bio-Rad Laboratories, UK). DL2000 ladder (TaKaRa Biotechnology, China) was used as the DNA molecular weight marker.

RESULTS AND DISCUSSION

Primer screening

Through preliminary screening, 20 of 53 primers were selected that amplified visible bands. Finally, 14 cpSSR primers that produced clear, reproducible, and polymorphic bands were selected with the proper annealing temperatures (Table 2; Figure 1). Amplifications of primers 9 and 10 were illustrated in Figure 1.

The annealing temperature is an important factor affecting PCR specificity; its role is to allow the primer and template to bind. Low annealing temperatures can ensure the stability of the combination of the primer and template (Powell et al., 1995), but when the annealing temperature is too low, the primer and the template can be mismatched, and non-specific bands can be generated. However, if the annealing temperature is too high, the binding of primer and template is inhibited, and the PCR products do not appear. Therefore, the optimal annealing temperature test is necessary when screening primers.

Table 1. Optimization of the cpSSR-PCR reaction parameters for three species of rhubarb.

	Mg ²⁺ concentration (mM)	Taq DNA polymerase (Units)	dNTPs concentration (mM)	Primer concentration (μM)	DNA template concentration (ng)	Annealing temperature (°C)
	0.5	0.25	0.04	0.10	5	56
	0.7	0.50	0.06	0.20	10	57
	1.0	1.00	0.08	0.30	15	58
	1.5	1.50	0.10	0.40	20	59
	2.0	2.00	0.20	0.50	30	60
	2.5	2.50	0.25	0.60	40	61
	3.0	-	0.30	0.80	50	-
	-	-	0.40	-	100	-
Optimum conditions	2.0	1.0	0.10	0.80	20	56-58

Table 2. Fourteen cpSSR primers selected.

Primer code	Gene names	Primer sequences (5'-3')	SSR motif	Optimal annealing temperature (°C)
1	Rpl2-Rpl23	F: GCT TAT GAC CTC CCC CTC TAT GC R: TGC ATT ACA GAC GTA TGA TCA TTA	(T) ₉	56
2	TrnL	F: TAC GAG ATC ACC CCT TTC ATT C R: CCT GGC CCA ACC CTA GAC A	(T) ₂ C(T) ₂	57
3	Rrn5-TrnR	F: CAC ACC AAT CCA TCC CGA ACT R: GGT GCG TTC CGR GGT GTG A	(A) ₁₃	58
4	Ycf5	F: TCG TTG GAT TTC TTC DGG ACA TTT R: CCC AAT ATC ATC ATA CTT ACR TGC	(A) ₈	56
5	Ycf5	F: CTA TGC AGC TCT TTT ATG YGG ATC R: TCC ARG TAA TAA ATG CCC AAG TT	(T) ₈	56
6	TrnR-Rrn5	F: CCA CCC CGT CTC SAC TGG ATC T R: AAA AAT AGC TCG ACG CCA GGA T	(T) ₁₃	57
7	TrnL-16SfRNA	F: CCG ACC TAG GAT AAT AAG CYC ATG R: GGA AGG TGC GGC TGG ATC	(T) ₈	56
8	Rpl2-TrnH	F: AYG GRG GTG GTG AAG GGA G R: TCA ATT CCC GTC GTT CGC C	(A) ₁₄	56
9	RpoB	F: CGA CAA TCC TTC CTA ATT CAC R: AGA AAA GMA AGG ATA TGG GCT C	(T) ₈	56
10	PsbC-TrnS	F: CGG GAA GGG CTC GKG CAG R: GTT CGA ATC CCT CTC TCT CCT TTT	(T) ₁₁	58
11	TrnK	F: TCA AAT GAT ACA TAG TGC GAT ACA R: AAT AAA GGA TTT CTA ACC ATC TT	(T) ₁₀	56
12	Rps2-RpoC2	F: TCT GAT AAA AAA CGA GCA GTT CT R: GAG AAG GTT CCA TCG GAA CAA	(T) ₁₀	56
13	Ycf3	F: GAG GAT ACA CGA CAG ARG GAR TTT R: CCT ATT ACA GAG ATG GTG YGA TTT	(A) ₁₃	57
14	Rps19-Rpl2	F: GGG TAT AAT GGT AGA TGC CC R: GCC GTA GTA AAT AGG AGA GAA A	(T) ₁₄	56

**Figure 1.** Amplification result of cpSSR by primers 1, 3, 9, and 10 by using different annealing temperature. Lane M = marker; lanes 1-6 = Primer 1, 56-61°C; lanes 7-12 = Primer 3, 56-61°C; lanes 13-18 = Primer 9, 56-61°C; lanes 19-24 = Primer 10, 56-61°C.

Optimization of the Mg²⁺ concentration

It has been reported that the PCR yield rate was subject to the Mg²⁺ concentration. When the Mg²⁺ concentration was below 0.5 mM or above 2.0 mM, there were no amplification products. The largest yield was obtained when the concentration was 2.0 mM (Figure 2).

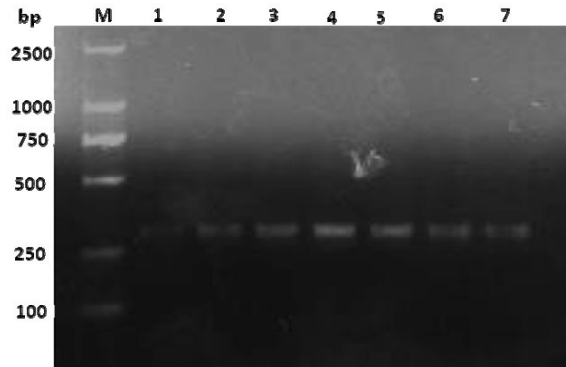


Figure 2. Amplification result of cpSSR with different concentration of MgCl₂ by primer 2. Lane M = marker; lanes 1-7 = 0.5, 1.0, 1.5, 2.0, 2.25, 2.5, 3.0 mM.

Mg²⁺ is the activator of *Taq* DNA polymerase, and its concentration has an important effect on the enzyme activity, primer specificity, and PCR amplification efficiency. If the Mg²⁺ concentration is too high, non-specific amplification products would be generated easily, and background interference is strengthened. Meanwhile, an Mg²⁺ concentration that is too low would reduce the amplification products, possibly resulting in no amplified products (Zhen et al., 2007).

Optimization of *Taq* DNA polymerase

PCR products significantly increased when the amount of *Taq* DNA polymerase reached 1.0 U. In order to reduce the cost of experiments, 1.0 U *Taq* DNA polymerase was suggested as the optimum amount (Figure 3).

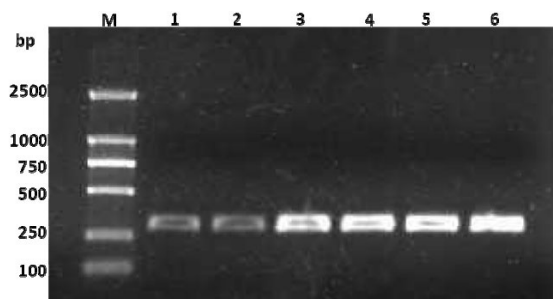


Figure 3. Amplification result of cpSSR with different amount of *Taq* DNA polymerase by primer 4. Lane M = marker; lanes 1-6 = 0.25, 0.50, 1.00, 1.50, 2.00, 2.50 U.

Taq DNA polymerase is also an important factor that affects PCR amplification. Excessive *Taq* DNA polymerase increases not only the cost of experiments but also the number of non-specific amplification products. Of course, too little *Taq* DNA polymerase reduces the amount of PCR products (Zhen et al., 2007).

Optimization of the primer concentration

When the primer concentration increased, the yield of PCR products was also enhanced. As illustrated in Figure 4, there were good amplification bands at the 0.5, 0.6, and 0.8 μM primer concentrations, and the most PCR product was harvested at 0.8 μM . Thus, we can select different primer concentrations according to the experimental purposes. For example, we can use the 0.5 μM primer concentration in the detection test in order to reduce the cost, while 0.8 μM can be used to obtain more PCR products.

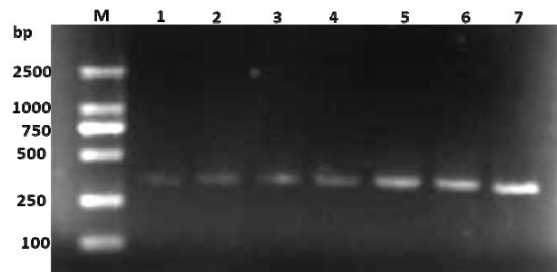


Figure 4. Amplification result of cpSSR with different concentration of primer by primer 2. Lane M = marker; lanes 1-7 = 0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.80 μM .

The binding capacity of the primer with the template is a main factor that affects the amount and the stability of amplification products. Primer concentrations that are too high cause non-specific amplification, and primer concentrations that are too low result in an insufficient amount of PCR product, which can affect the detection of PCR products.

Optimization of the dNTP concentration

As revealed by Figure 5, the PCR yield reached its maximum when the dNTP concentration was 0.1, 0.2, and 0.25 mM. If the concentration was higher than 0.25 mM, the PCR yield decreased. In order to reduce the experiment cost, the optimal dNTP concentration was 0.1 mM.

dNTPs are the raw materials of the amplified target sequence. If the dNTP concentration was higher than 0.25 mM, the activity of *Taq* DNA polymerase was inhibited (Zheng and Lu, 2003). When the dNTP concentration was too low, the PCR ended prematurely. Therefore, the optimal concentration should be selected.

Optimization of DNA template

Figure 6 showed that the PCR yield was subject to the DNA template concentration. In this experiment, PCR products increased as the DNA template concentrations increased,

but there was not a strictly positive relationship between them. The PCR yield rate did not increase when the DNA template amount was 20 ng. Therefore, we recommended that 20 ng DNA template is suitable and economic.

The quality of DNA template is very important for PCR amplification. Protein or residual impurities in the template inhibit the amplification.

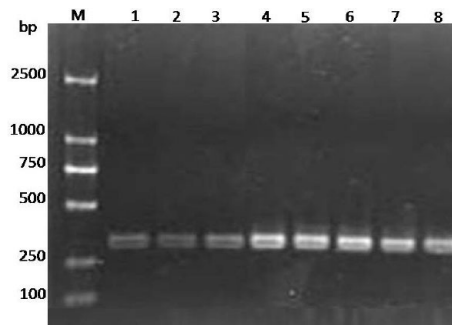


Figure 5. Amplification result of cpSSR with different concentration of dNTP by primer 6. Lane M = marker; lanes 1-10 = 0.04, 0.06, 0.08, 0.10, 0.20, 0.25, 0.30, 0.40 mM.

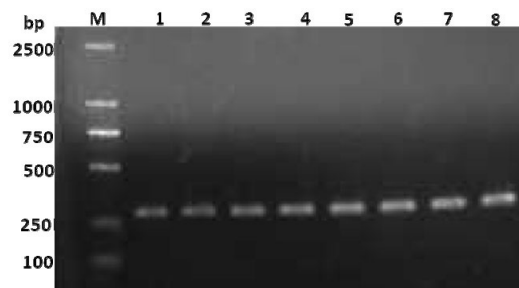


Figure 6. Amplification result of cpSSR with different concentration of template DNA by primer 8. Lane M = marker; lanes 1-8 = 5, 10, 15, 20, 30, 40, 50, 100ng.

CONCLUSION

In this study, the optimal cpSSRs-PCR conditions were obtained for a total reaction volume of 25 μ L: 20 ng DNA template, 0.8 μ M primers, 1 U *Taq* DNA polymerase, 2.0 mM Mg^{2+} , and 0.1 mM dNTPs. Additionally, the best annealing temperature ranged from 56 to 58°C for each primer. According to our optimal conditions, 14 of 53 primers were chosen for their clarity and repetition in three species.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (#81001602).

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