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Comparison of methods to preserve *Rheum palmatum* (Polygonaceae) for efficient DNA extraction and PCR amplification

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ABSTRACT. In this study, we compared the quality of DNA extracted using the modified CTAB method, from *Rheum palmatum* leaves preserved using fourteen different methods, including ones used commonly in other species: under ultra-cold (-80°C) temperatures, after drying with an absorbent paper, desiccating using a silica gel, drying at 60°C, in 70% ethanol, absolute ethanol, 70% ethanol supplemented with 50 mM EDTA, SDS-DNA extracting solution, nuclear separation buffer, improved NaCl-CTAB solution, TE-buffer, I-solution, or II-solution. DNA extracted from fresh leaves was used as the control. The quality of extracted DNA was evaluated based on the success of PCR amplification of the ITS2 region and a microsatellite marker. DNA was not extracted from samples preserved in the nuclear separation buffer and II-solution. The purities of DNA extracted from leaves preserved in ultra-cold temperatures, 70% ethanol, and 70% ethanol with 50 mM EDTA, and after desiccating using a silica gel and drying were higher, and comparable to the purity of DNA extracted from fresh leaves, than

those of leaves preserved using other methods. In the present study, combined with the PCR amplifications, the preservation using ultra-cold temperatures, silica gel desiccation, or drying, and PCR amplification of the extracted DNA can be used for further molecular studies in *R. palmatum*.

Key words: DNA extraction; PCR-amplification; Preservation; *Rheum palmatum*

INTRODUCTION

Considerable improvements in DNA sequencing technology, specifically the recent developments in next-generation sequencing technologies, and concomitant reductions in its cost have been driving factors for the widespread and routine use of molecular markers in conducting studies on population genetics, phylogenetics, phylogeography, ecology, and conservation genetics in all types of organisms (Holsinger, 2010; Carrió and Rosselló, 2014). *Ex situ* sample collection is the need of the hour for population-based studies; in fact, plant samples are always collected over an extensive distribution range and from remote fields. Fundamentally, analys must ensure that the detectable diversity of these studies is maximized by optimizing the quality and quantity of analyzed DNA and minimizing the bias of the methods. A major bias occurring in molecular studies is caused by the method used to preserve DNA samples.

Therefore, the methods used to store plant samples in the field or in biological repositories prior to DNA extraction are very important, as poor storage can lead to DNA degradation, as well as co-precipitation of PCR inhibitors.

The most effective method of sample storage is freezing the live samples at ultra-cold (-80°C) temperatures. However, traditional approaches for sample storage and transportation, including storing in liquid nitrogen or dry ice, may not be suitable for use in remote sites. Other methods, such as transporting samples in liquid fixatives (e.g., ethanol and other buffers), are also subject to many risks, such as leakage in the field or during transport, difficult customs policies in certain countries (Gemeinholzer et al. 2010; Carrió and Rosselló, 2014). To date, the most popular and widely-used method for the collection and storage of plant samples with the goal of obtaining high-quality DNA is to dry specimens with silica gel in polyethylene bags (Chase and Hills, 1991). However, a considerable amount of silica gel must be carried, as silica gel repositories are not readily available in remote areas, where the most endangered species are located. Thus, the use of silica gel may not be very cost-effective for the long-term storage of collected samples, and its use in developing countries with limited research budgets could compromise larger projects involving the national conservation of genomic resources.

Rheum palmatum L. is a perennial herbaceous rhubarb plant belonging to the family Polygonaceae; the rhubarb species *R. palmatum*, *Rheum officinale* Baill., *Rheum tanguticum* (Maxim. ex Regel) Maxim. ex Balf are endemic to China. The dried roots and rhizomes of these three species are officially used in Chinese medicine (Da-huang in Chinese) (Chinese Pharmacopoeia Committee, 2010). Da-huang has been used as an important compound in a number of Chinese patents or traditional medicines to cool blood detoxification, expel stasis pass-through, remove dampness, and abate jaundice, etc., for over 2000 years (Tseng et al.,

2006; Huang et al., 2007; Shia et al., 2009; Chinese Pharmacopoeia Committee, 2010). The huge demand for official Da-huang has resulted in excessive consumption of the plants; as a result, *R. tanguticum* has been listed in the official Chinese registry of endangered higher plants (Chen et al., 2009; Hu et al., 2010), and the existence of two other species is believed to be 'threatened' in China. *R. palmatum* has the widest distribution range among the three species, and grows in the Shanxi, Shaanxi, Gansu, Sichuan, and Qinghai provinces and the Tibetan Autonomous Region in China, at altitudes of 1000-5100 m above sea level. DNA molecular markers in medicinal plants have been used to provide fundamental information to aid in the future conservation, cultivation, and management of important medicinal species.

In this study, the quality of DNA extracted from *R. palmatum* samples preserved using fourteen different methods. The DNA quality was assessed according to the yield and purity of extracted DNA. The suitability of this DNA as a template for amplification was evaluated by the results of a PCR amplification of its ribosomal second internal transcribed spacer (*ITS2*), a candidate DNA barcode and microsatellite marker that is used to study genetic variation.

MATERIAL AND METHODS

Materials and storage procedures

Fresh leaves of *R. palmatum* were collected from a single plant grown in the medical garden of Xi'an Jiaotong University (previously transplanted from Zhuque National Forest Park, Hu County, Shaanxi, China). DNA was extracted from the leaves stored as follows: fresh leaves (not stored; sample 1); stored (stored samples 2-14) for 15 days in an ultra-cold refrigerator (-80°C); stored by drying with an absorbent paper, desiccating using a silica gel, or drying at 60°C; or stored in 70% ethanol, absolute ethanol, 70% ethanol supplemented with 50 mM EDTA (Chen et al., 2013), SDS-DNA extracting solution (Wang and Huang, 2002), nuclear separation buffer (Li et al., 2006), improved NaCl-CTAB solution (Rogstad, 1992), TE-buffer, I-solution (Qi, 2004), or II-solution (Qi, 2004) (Table 1). Each sample was duplicated 5 times.

Table 1. Fourteen preservation methods used in this study.

Sample No.	Preservation method
1	Fresh
2	Freezing at ultra-cold (-80°C) temperatures
3	Drying using absorbent paper
4	Desiccation by silica gel
5	Drying at 60°C
6	Storage in 70% ethanol
7	Storage in absolute ethanol
8	Storage in 70% ethanol with 50 mM EDTA
9	Storage in SDS-DNA extracting solution
10	Storage in nuclear separation buffer
11	Storage in improved NaCl-CTAB solution
12	Storage in TE-buffer
13	Storage in I-solution
14	Storage in II-solution

Reagents

The following reagents were used for sample storage and DNA extraction: SDS-DNA

extracting solution (2% SDS, 500 mM NaCl, 50 mM EDTA, 100 mM Tris-HCl), pH 8.0; nuclear separation buffer (200 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 250 mM NaCl); improved NaCl-CTAB solution (saturated NaCl; 2% PVP; 3-4% CTAB; 50 mM EDTA, pH 8.0; 2% β-mercaptoethanol); TE-buffer (10 mM Tris-HCl buffer, pH 8.0; 1 mM EDTA, pH 8.0); I-solution (0.4 M glucose, 3% PVP, 2% β-mercaptoethanol); II-solution (100 mM NaAc, 50 mM EDTA-Na_2, 500 mM NaCl, 2% PVP, 1.4% SDS, 2% β-mercaptoethanol; pH 4.8); extraction buffer I [0.2 M Tris-HCl pH 8.0; 0.05 M EDTA, pH 8.0; 0.25 M NaCl, 1% β-mercaptoethanol (w/v)]; and extraction buffer II [0.1 M Tris-HCl, pH 8.0; 0.02 M EDTA, pH 8.0; 1.4 M NaCl, 2% CTAB (w/v), 0.3% β-mercaptoethanol (w/v)].

DNA extraction

The genomic DNA of *R. palmatum* was extracted using a modified CTAB method. Dried leaves of *R. palmatum* (approximately 0.1 g) were ground in liquid nitrogen using a mortar and pestle, along with small amounts of quartz sand, ascorbic acid (1/10 the weight of dried leaves), and PVP powder (1/10 the weight of dried leaves). The powder was transferred to a 2-mL centrifuge tube and mixed with 2 mL cold extraction buffer I, containing 10 µL 2-mercaptoethanol (added just before use). The mixture was incubated at 0°C for 30 min and subsequently centrifuged at 1500 *g* for 10 min at 4°C. The supernatant was discarded and the precipitate was suspended in 800 µL preheated extraction buffer II (10 µL 2-mercaptoethanol was added to buffer II prior to use). The mixture was incubated at 65°C for 60 min with gentle intermittent shaking. The mixture was then centrifuged at 11,600 *g* for 6 min at 4°C. The supernatant was carefully decanted and transferred to a new 2-mL centrifuge tube; an equal volume of chloroform:isoamyl alcohol (24:1) and one tenth volume of preheated extraction buffer II was added and gently mixed by inversion for 10 min. This mixture was centrifuged at 13,800 *g* for 10 min at 4°C. The aqueous layer was carefully transferred to a fresh tube and re-extracted with one tenth volume of preheated extraction buffer II and chloroform:isoamyl alcohol (24:1) by centrifuging at 13.8 x 10^3 *g* for 10 min (repeat the step 1-2 times). The upper aqueous layer was transferred to a new 1.5 mL centrifuge tube and two volumes of ice-cold isopropanol was added. This was then stored at -20°C for 60 min and subsequently centrifuged at 11.6 *g* for l0 min at 4°C. The DNA pellet was washed twice with 70% ethanol and air-dried. Finally, the DNA pellet was re-suspended in 100 µL sterilized double distilled water.

Determination of concentration and quality of extracted DNA

The extracted DNA was quantified using a ultraviolet (UV)-spectrophotometer (ND-2000; NanoDrop, Wilmington, DE, USA) at 260 and 280 nm. The purity of the extracted DNA was indicated by the A_{260}/A_{280} ratio. DNA concentration and purity were also determined by electrophoresis on a 1.0% agarose gel, by comparing the intensities of obtained bands against the 1-kb Plus DNA marker ladder. The re-suspended DNA was then diluted in sterilized ddH_2O to a concentration of 50 ng/µL, and stored at -20°C for further use as a template.

PCR amplification of nuclear and microsatellite markers

PCR amplification of the target genes is used as a measure of the quality of isolated

DNA. The success of PCR amplification is denoted by a visible band, indicating a fragment of the expected size, in a resolved agarose gel. Therefore, the entire *ITS2* region and a pair of microsatellite markers were used to evaluate the quality of the DNA extracted.

The *ITS2* region was amplified using a previously described pair of primers (Chen et al., 2010). A microsatellite primer developed using paired-end Illumina shotgun sequencing was also used in this study. SSR-PCR performed using the forward primer Rpa28-f (5'-GAAGCACCACCACCTCTT-3') and the reverse primer Rpa28-r (5'-TCATCCTCACACATACACAAC-3'). *ITS2* and SSR PCR were performed on a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The *ITS2*-PCR was performed in a 25-μL reaction mixture, containing 2X Taq MasterMix (Taq DNA Polymerase, 2X Taq PCR Buffer, 1.5 mM $MgCl_2$, and 200 μM dNTP mix; Cwbiotech, Beijing, China), 0.5 μM each of the forward and reverse primers, and approximately 50 ng template DNA. The reaction conditions were set as follows: initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 45 s; a final extension at 72°C for 10 min; and a hold temperature of 4°C at the end.

SSR-PCR was performed in a 20-μL reaction mixture, containing 2X Taq MasterMix (Taq DNA Polymerase, 2X Taq PCR Buffer, 1.5 mM $MgCl_2$, and 200 μM dNTP mix; Cwbiotech), 0.2 μM each of the forward and reverse primers, and approximately 50 ng template DNA. The reaction was set as follows: initial denaturation at 94°C for 5 min; 37 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; final extension at 72°C for 10 min, and eventual storage at 4°C.

All amplified products were electrophoresed on 1% agarose gels with 1X TBE Buffer at 110 V for 25 min, and stained with ethidium bromide (0.5 μg/mL). The gels were visualized and photographed in UV light using the Bio-Rad Gel Documentation System (Bio-Rad, Hercules, CA, USA). The DL2000 ladder (TaKaRa Bio Inc., Dalian, China) was used as the molecular weight standard.

RESULTS

Modified CTAB extraction of DNA from samples preserved by drying at 60°C resulted in the highest yield, followed by that of samples preserved in absolute ethanol. The samples preserved using the nuclear separation buffer and II-solution did not produce any viable DNA (Table 2). The average A_{260}/A_{280} ratio of 12 preservation methods ranged from 1.73-2.22. Moreover, DNA extracted from samples dried at 60°C, or preserved in absolute ethanol, improved NaCl-CTAB solution, and TE-buffer were highly degraded, and DNA extracted from samples preserved in SDS-DNA extracting solution and I-solution were also slightly degraded. Furthermore, impurities were clearly observed in several wells (wells 5, drying at 60°C; 7, storage in absolute ethanol; 11, in improved NaCl-CTAB; 12, TE-buffer; and 13, in I-solution), indicating that polysaccharides and other secondary metabolites were not removed. In brief, the purity of DNA extracted from leaves preserved under ultra-cold temperatures; after drying with absorbent paper or desiccating using a silica gel; or in 70% ethanol or 70% ethanol with 50 mM EDTA was higher and comparable to that of DNA extracted from fresh leaves, compared to that of DNA preserved using other methods (Table 2 and Figure 1).

Table 2. Concentration and purity of DNA extracted from *Rheum palmatum* leaves preserved using fourteen different methods.

Sample number	Concentration (ng/μL)	Purity (A_{260}/A_{280})
1	217.7	1.81
2	134.4	1.82
3	94.3	1.87
4	118.5	1.87
5	979.3	2.11
6	190.3	2.00
7	622.9	1.99
8	461.5	1.83
9	221.0	2.22
10	0	0
11	182.8	1.73
12	213.4	1.74
13	230.1	1.85
14	0	0

ITS2-PCR amplifications of DNA extracted from samples stored using different methods, except storage in 70% ethanol with 50 mM EDTA (well 8), TE-buffer (well 12), and I-solution (well 13), were successful and comparable to those of DNA extracted from fresh leaves (Figure 2). However, SSR amplification of DNA extracted from fresh leaves (well 1), and leaves stored at -80°C (well 2), by drying on absorbent paper (well 3), desiccating on a silica gel (well 4), drying at 60°C (well 5), or stored in 70% ethanol (well 6) and SDS-DNA extracting solution (well 9) (Figure 3) was successful, that is the expected amplicons were obtained after replication of this analysis.

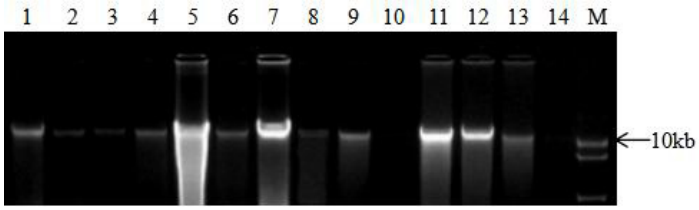

Figure 1. Electrophoretogram of DNA extracted from leaf samples stored using different methods. *Lane 1*, fresh leaves; *lane 2*, leaves stored in ultra-cold temperatures (-80°C); *lane 3*, leaves stored by drying; *lane 4*, desiccation using silica gel; and *lane 5*, storage by drying at 60°C; *lane 6*, leaves stored in 70% ethanol aqueous solution; *lane 7*, in absolute ethanol; *lane 8*, in 70% ethanol with 50 mM EDTA; *lane 9*, in SDS-DNA extracting solution; *lane 10*, nuclear separation buffer; *lane 11*, improved NaCl-CTAB solution; *lane 12*, TE-buffer; *lane 13*, I-solution; 14, II -solution; *lane M*, 1-kb Plus DNA ladder.

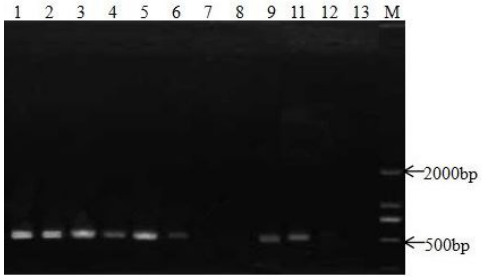

Figure 2. PCR amplification of ITS-2 region. The lanes indicate samples stored using different methods, as detailed in Figure 1. *Lane M*, 2000-bp DNA ladder.

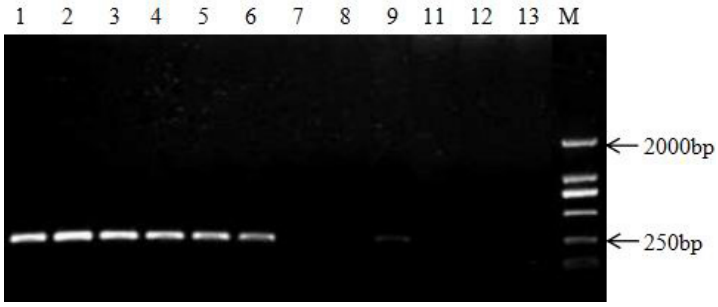

Figure 3. PCR amplification using a single SSR primer. The lanes indicate samples stored using different methods, as detailed in Figure 1. *Lane M* = 2000-bp DNA ladder.

DISCUSSION

Samples used for plant molecular studies are primarily collected from remote sites. Fresh plant samples, especially leaves collected from an individual plant, are known to wither and become rotten after a long transit time, resulting in the degradation of DNA by nucleases. Therefore, a stable, reliable, and economic method for the preservation of samples is an immediate necessity. A few studies have attempted to identify the best preservation method in different species; however, these studies have tested a very limited number of methods (Wang and Huang, 2002; Qi, 2004; Li et al., 2006; Chen et al., 2013; Carrió and Rosselló, 2014). In this study, we have analyzed all previously tested preservation methods to identify a few reliable and economic methods for the molecular study of rhubarb.

DNA was successfully extracted from *R. palmatum* leaves preserved for 15 days using 12 different methods, excluding the samples stored in the nuclear separation buffer and II-solution. The results revealed that the method used to preserve *Betula luminifera* (Li et al., 2006) for DNA extraction did not work for *R. palmatum*: although samples preserved by drying at 60°C provided the highest DNA yield, the extracted DNA was highly degraded, as seen in *Populus* leaves in a previous study (Liu et al., 2013). Our results further demonstrated that drying at high temperatures were not appropriate for the preservation of plant DNA.

The most reliable method used for the on-field preservation of plant samples so far is freezing in liquid nitrogen; however, this procedure has several disadvantages, including the lack of availability of liquid nitrogen in developing countries, as well as the difficulty in transporting and shipping the insulated, vented vessels (Chase and Hills, 1991). Therefore, we replaced liquid nitrogen with an ultra-cold freezer (-80°C) to preserve the *R. palmatum* leaves; we observed that the quality of DNA extracted from leaves stored at -80°C were comparable to that of DNA extracted from fresh leaves (Table 1 and Figure 1).

Silica gel desiccation is a fast and reliable method for the drying of plants; therefore, this method has been adopted by the scientific community worldwide, and is currently the standard tissue preservation method for molecular systematics and DNA barcoding (Carrió and Rosselló, 2014). Our results revealed that the quality of DNA extracted from *R. palmatum* leaves dried in silica gel was very high. However, this method also has a few disadvantages, such as the lack/low availability of silica gel repositories in remote areas. Additionally, the use of silica gel is not suitable for long-term storage, or for a large number, of samples. In this

study, samples were chiefly collected from higher altitude (1000-5000 m) areas; silica gel is usually used up quickly, and may not be sufficient for a 15-day preservation period, which could result in the onset of rot in some of the samples. Furthermore, *R. palmatum* leaves contain more water than the leaves of other species.

DNA was also extracted from *R. palmatum* leaf samples stored in different preservatives; however, the obtained DNA could not be successfully amplified by PCR. Moreover, the results of the *ITS2* and SSR amplifications were not comparable. *ITS2*-PCR was performed using DNA extracted from samples stored in 70% ethanol, SDS-DNA extracting solution, and improved NaCl-CTAB solution; however, the resultant bands showed a weak intensity. Furthermore, the SSR-PCR amplification of DNA extracted from leaves preserved in SDS-DNA extracting solution and improved NaCl-CTAB solution was unsuccessful. These results suggested that the integrity of DNA extracts were affected by the type of storage methods. Poor storage methods can result in DNA degradation as well as co-precipitation of PCR inhibitors, which further contributes to the failure of PCR.

In conclusion, storage of *R. palmatum* leaves under ultra-cold temperatures, or after silica gel desiccation or drying, effectively preserve the sample DNA for molecular studies. This method can also be applied for other *Rheum* species.

Conflicts of interest

The authors declare no conflict of interest.

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