An Improved Chloroplast DNA Extraction Procedure for Whole Plastid Genome Sequencing

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Abstract

Background: Chloroplast genomes supply valuable genetic information for evolutionary and functional studies in plants. The past five years have witnessed a dramatic increase in the number of completely sequenced chloroplast genomes with the application of second-generation sequencing technology in plastid genome sequencing projects. However, cost-effective high-throughput chloroplast DNA (cpDNA) extraction becomes a major bottleneck restricting the application, as conventional methods are difficult to make a balance between the quality and yield of cpDNAs.

Methodology/Principal Findings: We first tested two traditional methods to isolate cpDNA from the three species, Oryza brachyantha, Leersia japonica and Prinsepia utihis. Both of them failed to obtain properly defined cpDNA bands. However, we developed a simple but efficient method based on sucrose gradients and found that the modified protocol worked efficiently to isolate the cpDNA from the same three plant species. We sequenced the isolated DNA samples with Illumina (Solexa) sequencing technology to test cpDNA purity according to aligning sequence reads to the reference chloroplast genomes, showing that the reference genome was properly covered. We show that 40–50% cpDNA purity is achieved with our method.

Conclusion: Here we provide an improved method used to isolate cpDNA from angiosperms. The Illumina sequencing results suggest that the isolated cpDNA has reached enough yield and sufficient purity to perform subsequent genome assembly. The cpDNA isolation protocol thus will be widely applicable to the plant chloroplast genome sequencing projects.

Introduction

Chloroplasts (plastids) are plant organelles that contain a circular DNA containing ~130 genes with the size ranging from 72 to 217 kb [1,2]. cpDNAs of green plants are exceptionally conserved in their gene content and organization, providing sufficient information for genome-wide evolutionary studies. Recent efforts have proven their potentials in resolving phylogenetic relationships at different taxonomic levels and understanding structural and functional evolution by using the whole chloroplast genome sequences [3,4,5].

Plant cpDNAs have been set as targets among the very early genome sequencing projects owing to their small sizes [6]. To date, at least 200 plant complete cpDNAs have been sequenced (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=2759&opt=plastid), and in the recent years, the number is rapidly increasing due to an extensive application of the second-generation sequencing technologies to the whole chloroplast genome sequencing. Despite its short sequence reads, excess sequence data produced by the second-generation sequencing technologies are fairly suitable for the genome assembly, because the chloroplast genome is much smaller in size and simple in structural complexity compared to nuclear genomes [7]. For example, a single 600 Gbp per run in the Illumina HiSeq-2000 (http://www.illumina.com) could conceivably sequence ~40,000 average-sized chloroplast genomes to a depth of 120×. Next-generation sequencing technologies have undoubtedly made it possible to sequence the entire plant genomes more efficiently and economically than ever before with decreased time and costs compared with traditional approaches [6]. With rapid progress in sequencing technologies, the acquisition of high quality cpDNAs from plant tissues for the whole genome sequencing is urgently needed.

Two experimental methods are often employed to collect cpDNAs in plants. The first is the whole chloroplast genome amplification from total DNA using long polymerase chain reaction (PGR), and the second is direct isolation of cpDNAs.
from fresh plant materials based on sucrose gradient. The former method is PCR-based cpDNA sequencing, which is usually used to the situation that substantial plant leaf materials (e.g., ~20 to 100 g fresh leaves) are unavailable but can be substituted by extracting total DNA from limited materials. The cpDNA fragments are further amplified by using the conservative primer pairs [8]. The latter focuses on isolating the chloroplasts from fresh plant leaves according to sucrose gradient centrifugation, followed by a direct extraction of cpDNAs from intact chloroplasts [9]. Of them, sucrose gradient centrifugation is limited by the availability of ultracentrifuges which are not facilitated in many laboratories [10]. As a result, the PCR method is the most extensively used among the chloroplasts sequencing projects regardless of its time-consumption and higher costs [11]. As an alternative of the sucrose gradient centrifugation process, DNAse I treatment [12] and high salt precipitation [13] have succeeded in isolating cpDNAs of some specific plant species, but further applications to additional species have been restricted, as both of them were not easy to make a balance between enough yield and quality with limited contamination of nuclear and mitochondria DNA [14].

The rapid progress in the next-generation technologies requires developing new methods to isolate cpDNAs with increased quality and yield, especially aiming to simplify the isolation process so as to meet the need for the whole chloroplast genome sequencing. We modified the above-described methods [9,12,13] to develop a new protocol and further applied it to isolate cpDNAs from the three species, *Oryza brachyantha, Leersia japonica* and *Prinsepia utihis*. To test their purities, these three isolated cpDNAs were subsequently sequenced by using the Illumina (Solexa) sequencing-by-synthesis technology.

**Results and Discussion**

**The isolation of cpDNA**

The cpDNA isolation includes the three basic steps: separation of plastids from leaf tissues, lysis of the chloroplasts, and purification of DNA. Because the isolation of intact chloroplasts often acts as a critical stage of the whole procedure, the method based on sucro gradient ultracentrifugation is the most commonly employed to effectively separate nuclear DNAs from cpDNAs. Using two grass species (*O. brachyantha* and *L. japonica*) and one rosid plant (*P. utihis*), we first performed cpDNA isolation by following the previously described procedure [9]. Electrophoresis of the resulting DNA displayed a very weak band on agarose gel, indicative of very low cpDNA yield (Figure 1C). This study only used 20 g fresh leaves, while more than 100 g of leaf tissues were recommended [9]. Another possible explanation was that, after the sucro gradient centrifugation, only a small amount of chloroplast pellet was collected, leading to the extraction of few cpDNAs. Because the library preparation for the whole genome sequencing needs a substantial amount of starting DNA, either repeated cpDNA isolation or substantial leaves are required to use this method. Considering the amount of time-consumed by sucro gradient preparations, two alternative methods, DNAse I treatment and high salt method, may be suitable to replace the sucro gradient centrifugation method.

The DNAse I treatment method used DNAse I to digest nuclear DNA that adheres to the outer chloroplast membrane. The success in isolating the cpDNAs was reported from two of many species which they have attempted [9]. When the three plant species were used in this study, however, we failed to isolate intact cpDNAs since they all were degraded by the DNAse I (Figure 1B). The result is consistent with the fact that DNAse I digest not only the nuclear DNA but also the cpDNA which might not be well protected within intact plastids [9,15].

The second alternative method employs a high NaCl concentration in the isolation buffers, which do not involve any sucro gradient centrifugation. This method was only reported to have succeeded in isolating the pea cpDNA [13]. Considering that only increasing the NaCl concentration may not be enough to enhance cpDNA purity, we made several modifications of the method to broaden its application to as many taxa as possible. The final protocol (see materials and methods) demonstrated the advantage of isolating sufficient cpDNAs with leaf materials of the same three plant species (Figure 1A).

As a modification of the sucro gradient centrifugation, the high salt method significantly simplified the cpDNA isolation process. By using this method, our first effort to isolate the cpDNAs also seems successful, as it can get a relatively clearly defined DNA band. When we increased the amount of fresh leaves, however, a positive correlation between increased DNA yield and the possibility of DNA degradation was found, indicating that there is more contamination of nuclear DNAs (Figure 2). The observation suggests that this method may not be suitable to isolate cpDNAs with high purity. As an alternative approach in chloroplast isolation, four to six volumes (v/w) cold isolation buffer (in the original protocol) may not be enough to homogenize the fresh leaves (e.g., 20 g fresh leaves with 100 ml isolation buffer). Therefore, we increased the amount of isolation buffer from 5 to 20 volumes of fresh leaves (e.g., 20 g fresh leaves with 400 ml buffer A in our protocol) in the subsequent experiment. Even when 50 g fresh leaves were used, a well-defined cpDNA band can be observed (Figure 2), suggesting that the modification led to a successful isolation of the cpDNAs. It is likely that about 20 g leaves may be more optimal as it could include less contaminating nuclear DNA. Furthermore, two additional centrifugation steps (200 g 20 min and 3500 g 20 min, separately) were used to discard the cell debris and collect chloroplast pellet. To decrease the nuclear DNA contamination that adheres to the outer chloroplast membrane, we also incorporated extra steps to wash...
the chloroplast pellet with buffer B, further increasing the purity of isolated cpDNAs. Last but not least, chloroplasts were lysed using SDS and Proteinase K instead of cetyltrimethylammonium bromide (CTAB) followed by phenol/chloroform extraction. The final isolated cpDNAs were digested with HindIII and the result was visualized on a 0.8% agarose gel (Figure 3). Among these modifications, incorporated gradual centrifugation steps were of the most importance, because they are able to increase the cpDNA purity by separating the chloroplasts from cell debris. If larger amounts of starting materials (e.g., 50 g fresh leaves) were used, it is necessary to add a second centrifugation step at 200 g.

Of these four methods, our modified high salt method was more efficient to isolate the cpDNAs, and most importantly, to balance cpDNA yield and purity to the greatest extent. Indeed, our lab has been employing this improved protocol and extracted hundreds of plant species, which has proved to be highly efficient to isolate cpDNA from more taxa of plants (unpublished data).

Sequencing chloroplast DNAs using the second-generation illumina sequencing technology

The vast improvements made in DNA sequencing technologies offer unprecedented opportunities to perform phylogenomic studies based on the whole chloroplast genome sequences. Multiplex sequencing with the second-generation technology allows multiple samples to be sequenced in a run, generating millions of reads that significantly increase the sequence depth [16]. To test the cpDNA purity isolated by our protocol, in this study, we sequenced these three chloroplast genomes by using Illumina sequencing technology. Sequencing reactions generated a total of 330 Mbp sequence data with 5 Mbp in O. brachyantha, 21 Mbp in L. japonica and 304 Mbp in P. utihis (table 1). A reference-guided chloroplast genome assembly was performed to roughly estimate the genome coverage (figure 4), the O. brachyantha (Figure 4A) and L. japonica (Figure 4B) were assembled to O. nivara, while P. utihis (Figure 4C) was assembled to Prunus persica.

We surprisingly found that the cpDNA purity, represented by the percentage of the reads aligned to the reference genome, were relatively consistent across the three species, although the amount of sequence data varied greatly among them, ranging from 51, 606 reads in O. brachyantha to 3, 132, 702 reads in P. utihis. The cpDNA reads were 51.6% in O. brachyantha, 43.0% in L. japonica, and 44.2% in P. utihis, respectively (table 1). The average coverage was only 19× in O. brachyantha, as only 5 Mbp were obtained. In P. utihis, however, the generation of 304 Mbp led to cpDNA genome coverage of 855.7× (table 1). In this study, all of the reference genomes were sufficiently covered, showing two peaks in the invert
repeat (IR) regions (figure 4). Despite the relatively fewer
sequences and thus lower genome coverage in 
O. brachyantha
there were no large gaps found in the consensus sequence
(Figure 4A). Our results thus suggest that, given the cpDNA purity
isolated with this modified method, obtaining 50 Mbp of sequence
data could lead to at least 100× average coverage of the
chloroplast genome which is sufficient for the assembly.

Previous studies [17,18] suggested that no more than 5% of
cpDNAs usually exists among the total DNA in angiosperms.
However, our protocol can efficiently isolate the cpDNAs with
percentages of about 40–50% (table 1). The RCA-based (rolling
circle amplification) cpDNA sequencing method [9] reported that
approximately 10–40% of the resulting RCA products consisted of
non-cpDNA [19]. In comparison, our method apparently showed
its power in isolating cpDNAs with improved quality and lowered
sequencing costs, although there is room to further improve the
cpDNA purity.

In conclusion, this study provides a quick and efficient method
for isolating cpDNAs from angiosperms. In comparisons with the
commonly used methods of sucrose gradient centrifugation and
the DNase I treatment, our modified method indeed works
competently when testing with leaf materials of the same three
plant species of O. brachyantha, L. japonica and P. utihis. The cpDNA
bands could be clearly defined on the agarose gel. By means of the
next-generation Illumina sequencing technology, the three isolated
cpDNA samples were subsequently sequenced and their purity
reached ~40–50%, which were sufficiently pure to further
perform the genome assembly. In addition, we tested the genome
coverages influenced by the sequence data, showing that only
~50 Mbp could attain at least 100× average coverage of the
chloroplast genome when the cpDNA purity reached ~40–50%.
In all, this modified method is able to serve as an efficient cpDNA
extract procedure to complete the chloroplast genome sequencing
of angiosperms.

Materials and Methods
Plant materials
The O. brachyantha and L. japonica (Poaceae) plants were grown in
the greenhouse, while P. utihis (Rosaceae) was transplanted in

Table 1. Summary of total sequenced data and aligned reads of three plant species.

| Species       | Total bases (bp) | Total reads | Aligned reads | Aligned (%) | Average coverage | Reference genome    |
|---------------|------------------|-------------|---------------|-------------|------------------|---------------------|
| Oryza brachyantha | 4 956 842       | 51 606      | 26 659        | 51.6        | 19.0             | O. nivara NC_005973 |
| Leersia japonica    | 21 321 958      | 221 544     | 95 268        | 43.0        | 69.2             | O. nivara NC_005973 |
| Prunsepia utihis     | 303 846 384     | 3 132 702   | 1 385 592     | 44.2        | 855.7            | P. persica NC_014697 |

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Botanical Garden of Kunming Institute of Botany, Chinese Academy of Sciences. For each plant species, ~20 g of the fresh leaves were collected and cleaned with distilled water, and then they were restored in 4°C refrigerator for further experimental uses.

**Protocols**

The four cpDNA isolation methods used in our study were described as below:

A. **Modified high salt method (Figure 5)**

**Reagents**

**Figure 5. Flowchart showing the major steps for the isolation of cpDNAs using the modified high salt method.**

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### Chloroplast isolation

All the following steps were carried out at 0°C if not otherwise stated.

1. Prior to extraction, about 20 g (fresh weight) leaves were collected and kept in dark for 48 to 72 hours at 4°C to decrease starch level stored in the leaves.

2. The leaves were nerve-removed, cut into pieces (~1 cm) and homogenized in 400 ml ice-cold buffer A for 30 seconds. Filter the homogenate into centrifuge bottles using two layers of Miracloth (Merck) by softly squeezing the cloth.

3. Centrifuged the homogenate (200 g, 20 min). The nucleus pellet and cell-wall debris were discarded.

4. Repeated the centrifugation once again. The supernatant included chloroplasts suspended in it.

5. Centrifuged the supernatant at a higher centrifugal force of 3500 g for 20 min, the resulting pellet were chloroplast pellet with some contamination of nuclear DNAs.

6. Added 250 ml Buffer B to the pellet and suspend it gently using a paintbrush to wash the nuclear DNAs attaching to the chloroplast cytomembrane. Then centrifuge with 3500 g for 20 min and discard the supernatant.

7. Re-suspended the pellet with 250 ml Buffer B again and centrifuged (3750 g for 20 min) to gain the purified chloroplasts.

### Chloroplast DNA isolation

8. Added 8 ml Buffer C, 1.5 ml 20% SDS, 20 μl β-Me, 30 μl Proteinase K (10 mg/ml) to the purified chloroplast pellet and incubate at 55°C for at least 4 hours or overnight. The chloroplasts would be fully lysed.

9. Put the centrifuge bottles on ice for 5 min, add 1.5 ml 1 M KAc (pH 5.2) and continue to freeze for 30 minutes. Then 10000 g 15 min, discarding the pellet.

10. Extracted the supernatant with an equal volume of saturated phenol and chloroform:isoamyl-alcohol (24:1) in the centrifugation of 10000 g 20 min for twice.

11. Treated the cpDNA sample with 2 μl RNAse and visualize the DNA band on a 0.8% agarose gel.

### Reagents

- **An Improved Chloroplast DNA Extraction Procedure**
- **An Improved Chloroplast DNA Extraction Procedure**

### Chloroplast genome sequencing and data analysis

After the cpDNA isolation with modified high salt method, approximately 3–10 μg of DNA was sheared, followed by adapter
ligation and library amplification, subjecting to Illumina Sample Preparation Instructions. The fragmented cpDNAs were sequenced at both single-read using the Illumina Genome Analyzer IIx platform at the in-house facility at The Germplasm Bank of Wild Species in Southwestern China. The obtained paired-end reads (2 × 100 bp read lengths) were assembled to the reference genome sequence to roughly estimate the genome coverage and cpDNA purity (the reads aligned to the reference genome sequence were served as cpDNA sequence) using the software program Geneious version 4.7 [20]. The reference chloroplast genome sequence of *O. nivara* (NC_005973) and *P. persica* (NC_014697) were downloaded from GenBank.

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**Author Contributions**

Conceived and designed the experiments: CS LG. Performed the experiments: CS JG HH NH. Analyzed the data: YZ. Contributed reagents/materials/analysis tools: CS YZ. Wrote the paper: CS LG.

**References**

1. Sugiura M (1995) The chloroplast genome. Essays Biochem 30: 49–57.
2. Sugiura M (1992) The chloroplast genome. Plant Mol Biol 19: 149–68.
3. Moore MJ, Bell CD, Solis PS, Solis DE (2007) Using plastid genome-scale data to resolve enigmatic relationships among basal angiosperms. Proc Natl Acad Sci USA 104: 19363–19368.
4. Jansen RK, Cai ZQ, Raubeson LA, Daniel H, dePamphilis CW, et al. (2007) Analysis of 81 genes from 64 plastid genomes resolves relationships in angiosperms and identifies genome-scale evolutionary patterns. Proc Natl Acad Sci USA 104: 19369–19374.
5. Moore MJ, Solis PS, Bell CD, Burleigh JG, Solis DE (2010) Phylogenetic analysis of 83 plastid genes further resolves the early diversification of eudicots. Proc Natl Acad Sci USA 107: 4625–4628.
6. Moore M, Dhingra A, Solis P, Shaw R, Farmerie W, et al. (2006) Rapid and accurate pyrosequencing of angiosperm plastid genomes. BMC Plant Biol 6: 17.
7. Shaffer C (2007) Next-generation sequencing outpaces expectations. Nat Biotechnol 25: 149.
8. Berthold Heinze (2007) A database of PCR primers for the chloroplast genomes of higher plants. Plant Methods 3: 4.
9. Jansen RK, Raubeson LA, Boone JL, dePamphilis CW, Chumley TW, et al. (2005) Methods for obtaining and analyzing whole chloroplast genome sequences. Methods Enzymol 395: 548–364.
10. Diekmann K, Hodkinson TR, Fricke E, Barth S (2008) An optimized chloroplast DNA extraction protocol for grasses (Poaceae) proves suitable for whole plastid genome sequencing and SNP detection. PLoS ONE 3: e2813.
11. Cronn R, Liston A, Parks M, Germandt DS, Shen RK (2008) Multiplex sequencing of plant plastid genomes using Solexa sequencing-by-synthesis technology. Nucleic Acids Res 36: e122.
12. Kolodner R, Tesarik KK (1979) Inverted repeats in chloroplast DNA from higher plants. Proc Natl Acad Sci USA 76: 41–45.
13. Beckjord G, Stummann BM, Henningsen KW (1984) Preparation of chloroplast DNA from pea plastids isolated in a medium of high ionic-strength. Anal Biochem 141: 244–247.
14. Lang BF, Burger G (2007) Purification of mitochondrial and plastid DNA. Nature Protoc 2: 652–660.
15. Palmer JD (1986) Isolation and structural analysis of chloroplast DNA. Methods Enzymol 118: 167–186.
16. Parks M, Cronn R, Liston A (2009) Increasing phylogenetic resolution at low taxonomic levels using massively parallel sequencing of chloroplast genomes. BMC Biol 7: 84.
17. Nock CJ, Waters DLE, Edwards MA, Bowen SG, Rice N (2010) Chloroplast genome sequences from total DNA for plant identification. Plant Biotechnol J. pp 1–6.
18. Tangphasomruang S, Sangyarak D, Champrasert J, Uthaipaisanwong P, Yoocha T (2010) The chloroplast genome sequence of mungbean (Vigna radiata) determined by high-throughput pyrosequencing: structural organization and phylogenetic relationships. DNA Res 17: 11–22.
19. Atherton RA, McComish BJ, Shepherd LD, Berry LA, Albert NW (2010) Whole genome sequencing of enriched chloroplast DNA using the Illumina GAI platform. Plant Methods 6: 22.
20. Drummond AJ, Ashton B, Cheung M, Heled J, Kearse M, et al. (2009) Geneious v4.7. Available from http://www.geneious.com.