

NaOH low-salt method for chloroplast isolation and highly pure cpDNA preparation from *Aeluropus littoralis*

Najmeh Nasiri^{1,5}, Ghorbanali Nematzadeh^{2,5}, Vahid Shariati-Joni³, and Hamid Najafi-Zarrini⁴

Manuscript received 4 May 2018; revision accepted 15 August 2018.

¹ Department of Molecular Plant Breeding and Genetic Engineering, University of Agricultural Sciences and Natural Resources, Sari, Iran

² Department of Plant Genetics, Genetic and Agricultural Biotechnology Institute of Tabarestan (GABIT), University of Agricultural Sciences and Natural Resources, Sari, Iran

³ Department of Plant Molecular Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

⁴ Department of Plant Breeding and Biotechnology, University of Agricultural Sciences and Natural Resources, Sari, Iran

⁵ Authors for correspondence: najmeh.nasiri@gmail.com, gh.nematzadeh@sanru.ac.ir

Citation: Nasiri, N., G. Nematzadeh, V. Shariati-Joni, and H. Najafi-Zarrini. 2018. NaOH low-salt method for chloroplast isolation and highly pure cpDNA preparation from *Aeluropus littoralis*. *Applications in Plant Sciences* 6(9): e1183.

doi:10.1002/aps3.1183

PREMISE OF THE STUDY: High-yield pure chloroplast DNA (cpDNA) is necessary for whole genome sequencing. Chloroplast extraction with traditional high-salt methods causes damage to nuclei and destroys the integrity of organelles, which leads to high genomic contamination from the nucleus and mitochondria. To overcome this issue, we modified a traditional high-salt method to obtain a new approach called the NaOH low-salt method (NLS).

METHODS AND RESULTS: The NLS method is based on the mild alkaline lysis of plant cells, followed by homogenization with ultrasonic waves and fractionation under reduced osmotic pressure. Results showed that this modified protocol worked efficiently to extract the intact chloroplast from *Aeluropus littoralis* and other grasses to obtain high-quality pure cpDNA, which was confirmed by fluorescent microscopy, qPCR, and Illumina paired-end sequencing analysis.

CONCLUSIONS: Compared with high-salt methods, the NLS method has proven robust for extraction of intact chloroplasts and preparation of high-yield pure cpDNA from grasses.

KEY WORDS chloroplast extraction; cpDNA preparation; high-salt methods; Poaceae; whole genome sequencing.

In recent years, whole chloroplast DNA (cpDNA) sequencing has dramatically increased. As a consequence of advances in sequencing strategies and bioinformatics tools, the number of available complete chloroplast genomes is growing steadily (Shi et al., 2012; Do et al., 2013). To perform whole chloroplast genome sequencing projects, it is necessary to isolate high-quality cpDNA from the target species. High-quality cpDNA is DNA that is obtained from intact chloroplasts and has low nuclear and organellar genomic contamination. Pure cpDNA results in highly clean reads that can reduce complexity and speed up further analysis, especially in the case of de novo-assembly with short reads (Kingsford et al., 2010; Islam et al., 2013). Unfortunately, no general method exists that works well across diverse plant species. Therefore, species-specific protocols to increase the quality and yield of cpDNA and to simplify the isolation process are urgently needed.

Total genomic DNA amplification or chloroplast extraction approaches are often used to collect cpDNA. The chloroplast extraction strategy focuses on isolating the chloroplasts from fresh plant materials using sucrose gradient centrifugation (Jansen et al., 2005) or high-salt (HS) and modified high-salt (mHS) precipitation methods (Bookjans et al., 1984; Shi et al., 2012). The cpDNA is then further isolated from the extracted chloroplasts. However, sucrose

gradient centrifugation is limited by the availability of ultracentrifuges, and cpDNA extracted with the high-salt methods can be highly contaminated by nuclear and mitochondrial DNA (Lang and Burger, 2007; Diekmann et al., 2008). As an alternative to address these challenges, after high-salt precipitation, the chloroplasts were layered over a Percoll concentration gradient and centrifuged with regular instruments at high speed (Takabe et al., 1979; Seigneurin-Berny et al., 2008). This led to an intact chloroplast fraction that was clearly separated from damaged chloroplasts, nuclei, mitochondria, and other residues. However, a principal drawback to this approach is the resulting low yield of isolated cpDNA, which is not sufficient for chloroplast genome sequencing when using current next-generation sequencing platforms. To obtain the desired amount of isolated cpDNA, the starting plant materials must be increased to 25–100 g and extraction must be performed in high volumes, which is not possible for most samples and significantly increases the final time and cost (Shi et al., 2012).

The main purpose of this study was to extract intact chloroplasts from grasses and prepare pure cpDNA with minimal contamination by nuclear and mitochondrial genomes. As mentioned above, cpDNAs obtained using the HS and mHS methods (Bookjans et al., 1984; Shi et al., 2012) are highly contaminated with other genomic

DNAs. Therefore, we have modified the original HS method and developed a new protocol to extract intact chloroplasts and prepare pure cpDNA from the halophyte grass *Aeluropus littoralis* (Gouan) Parl. *Aeluropus littoralis*, which is considered to be a salt-tolerant plant, is an Iranian endemic monocot halophyte with notable biological characteristics, including the evolution of a series of mechanisms to maintain osmotic balance and enhance salt tolerance (Nasiri et al., 2012). We also tested our method on other Poaceae species and confirmed that it is effective in preparing cpDNA of high quality.

METHODS AND RESULTS

Extraction of chloroplasts and cpDNA isolation

Aeluropus littoralis seeds (Appendix 1) were sown in soil at high density and grown under long-day conditions (16 h/8 h) at 23–25°C in the Sari Agricultural Sciences and Natural Resources University (SANRU) greenhouse for two months. The leaf samples of other species were collected from the field (Appendix 1). Then, leaf samples

were kept under a prolonged dark period of 48 h to decrease the starch content, as heavy starch accumulation has been shown to prevent the isolation of intact plastids during extraction (Pongratz and Beck, 1978). *Aeluropus littoralis* chloroplasts were isolated according to the classical HS (Bookjans et al., 1984) and mHS (Shi et al., 2012) methods as described previously. For the HS method (Fig. 1), 50 g of samples were homogenized in a Waring blender (model 7010S; Waring Products Inc., Torrington, Connecticut, USA) using approximately 150 mL of buffer B (Table 1). The homogenate was filtered through Miracloth (Calbiochem, San Diego, California, USA) and centrifuged at 3000 × g for 20 min. Finally, the obtained pellets were resuspended in 10 mL of buffer D (Table 1). For the mHS method (Fig. 1), approximately 20 g of fresh leaves were homogenized in 400 mL of ice-cold buffer B for 30 s in a Waring blender. The homogenate was filtered using two layers of Miracloth, then centrifuged at 200 × g for 20 min. The nucleus pellet and cell wall debris were discarded and the centrifugation was repeated. Then, the supernatant was centrifuged at a higher force of 3500 × g for 20 min and the resulting pellet (chloroplast pellet) was suspended in 250 mL of buffer C (Table 1) and centrifuged at 3500 × g for 20 min. The pellet was then resuspended in 10 mL of buffer D (Table 1) and centrifuged (3750 × g for 20 min) to gain the purified chloroplasts.

As an alternative, we modified the HS and mHS methods as a new protocol called the NaOH low-salt (NLS) method (Fig. 1). Briefly, 15 g of biomass (fresh leaf) was subjected to a mild alkaline-thermal pretreatment (300 mL of buffer A, Table 1) for 40 min under shaking at 37°C. After pretreatment, the liquid was removed by filtration, and the residual biomass was washed thoroughly with deionized water until the pH reached 7.0. All of the subsequent manipulations were carried out on ice. The rinsed biomass was homogenized in 200 mL of pre-cold extraction buffer B (Table 1) by the ultrasonic probe (5 min, 200 W) at short periods (6 s exposure, 4 s rest). The resulting suspension was filtered twice through two layers of Miracloth and then centrifuged at 200 × g for 20 min to eliminate cell debris. To precipitate the released chloroplasts, the homogenate was centrifuged (3000 × g for 20 min) and the obtained pellet was resuspended in 200 mL of washing solution (buffer C; Table 1). Finally, after another pelleting at 3000 × g for 20 min, the chloroplasts were homogenized in 10 mL of buffer D (Table 1) and stored at 4°C. The final pellets obtained by these methods (HS, mHS, NLS) were analyzed to compare yields and intactness of chloroplasts. Chloroplast DNA and total DNA were isolated from extracted chloroplasts and fresh leaves, respectively, according to previously described methods (Appendix 2, protocol 1) (Dellaporta et al., 1983; Shi et al., 2012). The cpDNA (Fig. 2) and total DNA (Fig. S1A) samples were treated with RNase and visualized on a 0.8% agarose gel after staining with ethidium bromide. Statistical analysis was

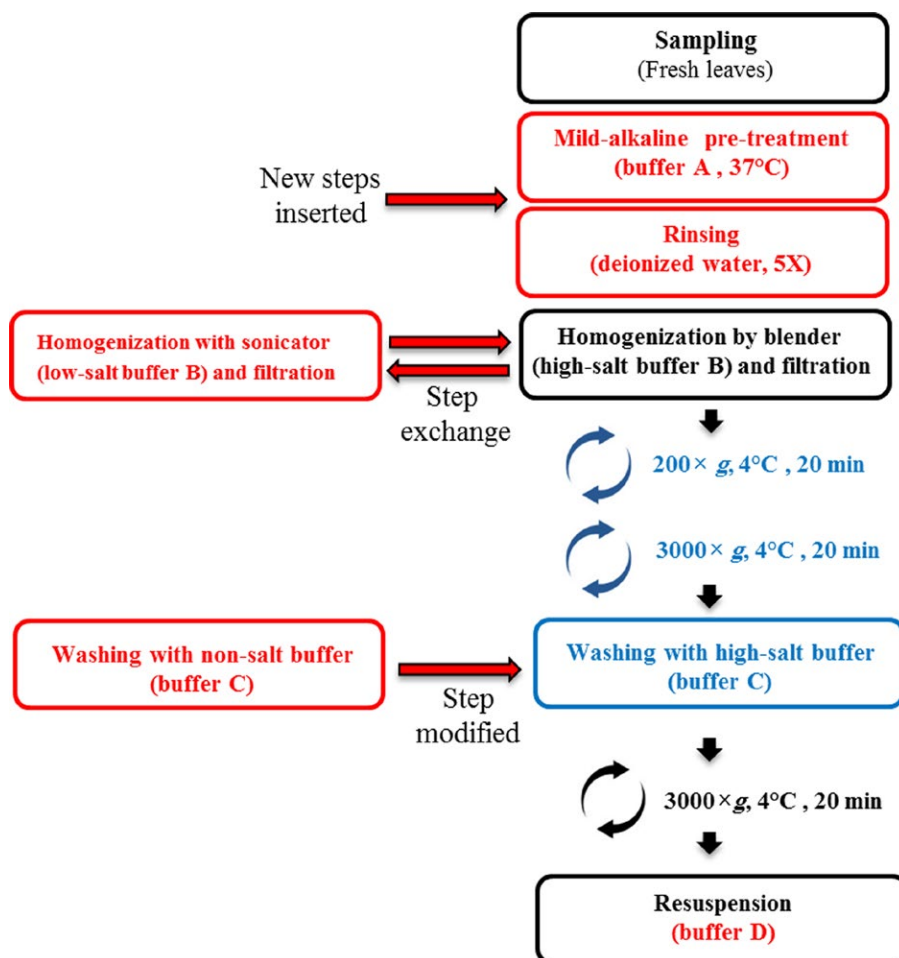


FIGURE 1. Diagram showing changes to classical chloroplast extraction schemes (high salt [HS] and modified high salt [mHS]) by insertion, exchange, and modification of the main steps and buffers to create a modified method (NaOH low-salt method [NLS]). Black, blue, and red boxes refer to the HS, mHS, and NLS protocols, respectively. Buffer A = alkaline lysis; buffer B = homogenization; buffer C = washing; buffer D = dilution (see Table 1).

TABLE 1. Reagents used in the high-salt (HS), modified high-salt (mHS), and NaOH low-salt (NLS) methods.

Method	Components (buffers ^a)	Reference
HS	NaCl (1.25 M), Tris-HCl (50 mM), EDTA (5 mM), BSA (0.1% w/v), β-ME (0.1% v/v) (buffers B and D)	Bookjans et al., 1984
mHS	NaCl (1.25 M), borax (0.0125 M), sodium metabisulfite (10 mM), Tris-HCl (50 mM), EDTA (7 mM), PVP (1%), DTT (1 mM), ascorbic acid (0.25 mM), BSA (0.1% w/v) (buffer B) NaCl (1.25 M), borax (0.0125 M), Tris-HCl (50 mM), EDTA (7 mM), PVP (1%), DTT (1 mM), BSA (0.1% w/v) (buffers C and D)	Shi et al., 2012
NLS	NaOH (0.5 M), EDTA (7 mM), PVP (1 g), β-ME (0.2% v/v) (buffer A) NaCl (0.62 M), Tris-HCl (50 mM), EDTA (7 mM), PVP (2 g), DTT (1.5 mM) (buffer B) Sorbitol (0.35 M), Tris-HCl (50 mM), EDTA (25 mM) (buffers C and D)	This study

Note: ascorbic acid = (5R)-[1S]-1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one; β-ME = 2-mercaptoethanol; borax = sodium tetraborate decahydrate; BSA = bovine serum albumin; DTT = dithiothreitol; EDTA = ethylenediaminetetraacetic acid; NaCl = sodium chloride; NaOH = sodium hydroxide; PVP = polyvinylpyrrolidone; sorbitol = (2S,3R,4R,5R)-hexane-1,2,3,4,5,6-hexol; Tris-HCl = tris hydrochloride.

^aBuffer A = alkaline lysis; buffer B = homogenization; buffer C = washing; buffer D = dilution.

done with SPSS Statistics 23 for Windows (IBM, Armonk, New York, USA). One-way ANOVA analysis with post-hoc pairwise comparisons by Fisher's Least Significant Difference (LSD) test was used to test for differences among experiments. A *P* value of less than 0.05 was considered statistically significant.

Purity assessment of cpDNA by qPCR

The purity level of cpDNA isolated from different chloroplast extracts (HS, mHS, NLS) and total DNA was analyzed via real-time PCR (qPCR) based on the gene copy number. For this, a nuclear-specific gene (*gigantea*), a unique chloroplast gene (*rbcl*), and one mitochondrial gene (*cox1*) were amplified per DNA sample. Primer sequences used in this study for qPCR analysis are shown in Appendix S1. The qPCR was carried out using Brilliant SYBR Green qPCR reagent (Stratagene, La Jolla, California, USA) with the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) as follows: denaturation at 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 20 s. The amount of nuclear and mitochondrial DNA (nDNA and mtDNA, respectively) in each sample relative to the number of chloroplast copies was determined by comparing the cycle threshold values according to the following formulas (Lutz et al., 2011), where nuE, mtE, and cpE are PCR efficiency (E) values of nuclear, mitochondrial, and chloroplast genes, respectively, and CT is the cycle threshold number.

The number of chloroplasts relative to the number of nuclear copies

$$= (\text{nuE}^{\text{gCT}}) / (\text{cpE}^{\text{cpCT}})$$

The number of chloroplasts relative to the number of mitochondrial copies

$$= (\text{mtE}^{\text{mtCT}}) / (\text{cpE}^{\text{cpCT}})$$

The copy number of studied genes amplified from each genome (chloroplast, nuclear, and mitochondrial) is shown in Fig. 3 and

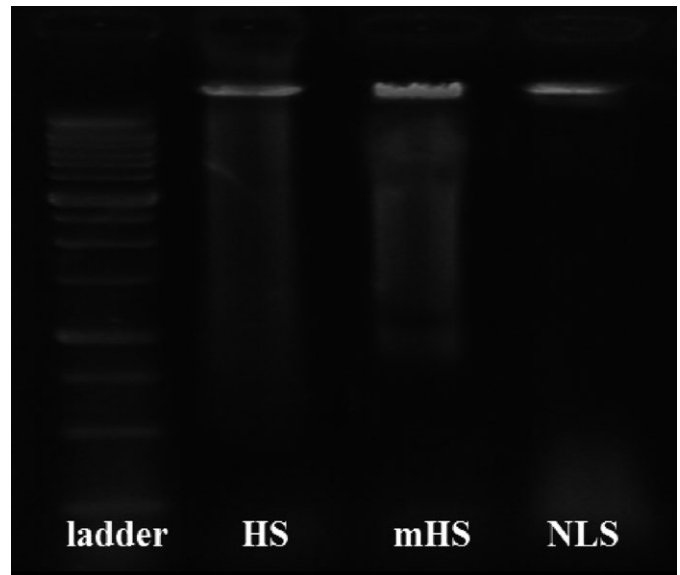


FIGURE 2. Isolated cpDNAs on agarose gel by three different chloroplast extraction methods (high salt [HS], modified high salt [mHS], and NaOH low-salt method [NLS]; 1-kbp DNA ladder [SM0311, Fermentas International Inc., Burlington, Ontario, Canada]).

based on that, the impurity percent of cpDNA with nDNA and mtDNA copies was calculated. The results showed that the most pure cpDNA preparation was achieved from NLS chloroplast extracts, where a significant reduction ($P < 0.05$) in both nDNA and mtDNA contaminations was seen in comparison with other methods. In this regard, the nuclei contamination with the NLS method was reduced to about 3.9×, 2.8×, and 6.8× compared with the HS, mHS, and total DNA methods, respectively (Fig. 3). In addition, based on the results, we found that the level of mtDNA contamination followed the order of total DNA > HS > mHS = NLS. In accordance with the qPCR results, the final DNA yield in each preparation also represents the level of contamination. The DNA concentrations based on the UV absorption (at 260 nm) were calculated to be 2130, 890, 1437, and 342 ng/mL for the total DNA, HS, mHS, and NLS methods, respectively (Table 2). Finally, the DNA bands on an agarose gel after electrophoresis showed no background smear in the NLS-derived cpDNA sample in contrast to HS- and mHS-derived cpDNAs, indicating that NLS-derived cpDNA was less contaminated than non-NLS-derived cpDNAs (Fig. 2).

Yield and intactness of extracted chloroplasts

To evaluate our procedure in comparison to basic methods, the yield and intactness of isolated chloroplasts from *A. littoralis* were measured by a spectrophotometer (Table 2) and visually analyzed under a microscope (Fig. 4). The yield of isolated chloroplasts was estimated based on the milligrams of chlorophyll per milliliter of the chloroplast suspension according to Arnon (1949) (Appendix 2, protocol 2). We found no significant difference by ANOVA or by Fisher's LSD ($P > 0.05$) between the mHS and NLS methods in terms of chlorophyll yield, whereas extraction using the HS method resulted in an insufficient amount of chloroplasts (Table 2).

To estimate the percentage of the intact chloroplasts, the rates of photoreduction of ferricyanide (Heber and Santarius, 1970)

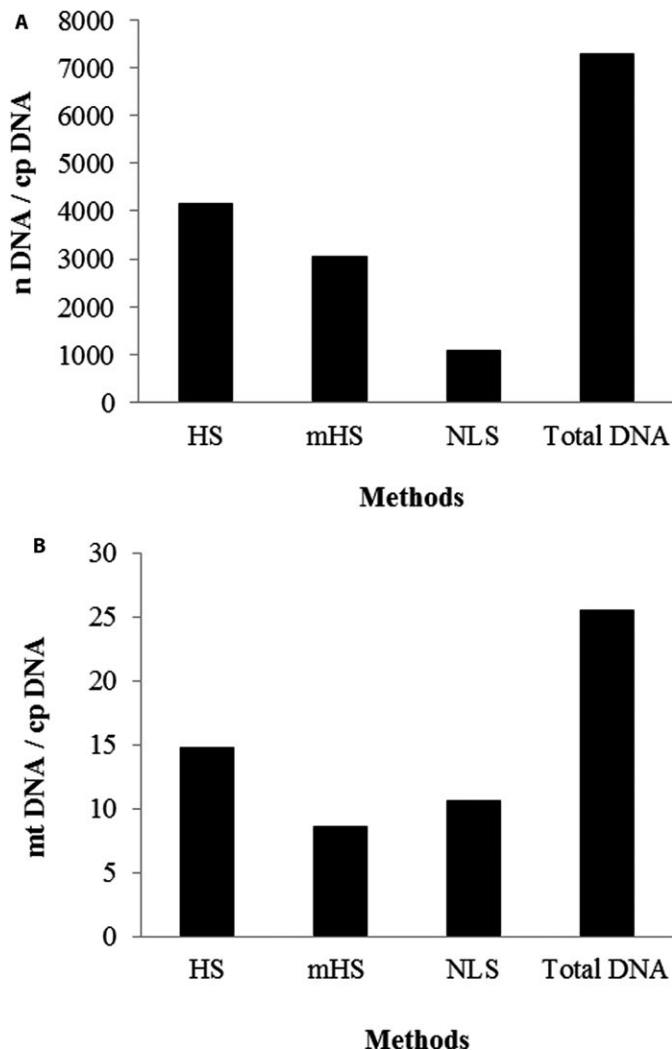


FIGURE 3. Comparison of cpDNA purity among different chloroplast isolation methods based on the relative expression level of the target genes (*gigantean*, *cox1*, *rbcl*). (A) Contamination of cpDNA with nDNA. (B) Contamination of cpDNA with mtDNA. HS = high-salt method; mHS = modified high-salt method; NLS = NaOH low-salt method.

and the rates of pyocyanin-catalyzed cyclic photophosphorylation (Lilley and Walker, 1973) were determined before and after osmotic shock (Appendix 2, protocol 3). The chloroplast integrity

was considerably higher using the NLS method (85.1%) than with the HS (49%) and mHS (61.2%) methods as determined by ferricyanide reduction (Table 2). Consistently, the maximal rate of pyocyanin-catalyzed cyclic photophosphorylation of exogenous adenosine diphosphate (ADP) by damaged chloroplasts (osmotically shocked chloroplasts) was obtained about 113.1, 458, and 288.2 $\mu\text{M mg}^{-1}$ chlorophyll h^{-1} for NLS, HS, and mHS preparations, respectively (Table 2). It has been shown that the rate of photophosphorylation increases after osmotic shock (Santarius, 1973). Thus, based on the relative rates of ferricyanide reduction and ADP phosphorylation, we showed that the proportion of intact chloroplasts in fractions extracted with the NLS protocol was significantly higher than in the HS and mHS methods as determined by ANOVA and Fisher's LSD at the 0.05 level (Table 2). In addition, brightfield and fluorescent microscopic images of isolated chloroplasts revealed that a large amount of broken chloroplasts (dark ruptured) could be observed in the extraction when using the HS and mHS methods (Fig. 4A). In contrast, chloroplast extracted using the NLS method appears bright green with a shiny halo of light under the phase contrast. Compared with broken chloroplasts as previously reported, intact chloroplasts are surrounded by a more pronounced halo (Fig. 4B). Furthermore, chlorophyll fluorescence analysis after carboxyfluorescein diacetate (CFDA) staining (Appendix 2, protocol 4) showed that chloroplasts that had taken up CFDA fluoresced intensely yellow or greenish yellow using nonspecific filters, owing to the presence of both green fluorescence and red autofluorescence of chlorophylls (Fig. 4A, B: i, ii, iii). In this case, yellow fluorescence could be detected only in low quantities of high-salt extracts, which indicates that the degree of chloroplast breaks was relatively high (Fig. 4A: iii). Finally, for a visual comparison of the intact chloroplast bands, the isolated chloroplasts were centrifuged on a Percoll density gradient (40%) according to the protocol of Mullet and Chua (1983). Our results were consistent with those of Mullet and Chua (1983), with the chloroplast fraction obtained using the NLS method showing a thinner upper band (indicative of broken chloroplasts) when purified on a continuous Percoll gradient in comparison to other methods (Fig. 4C).

Applicability of the NLS method in other grasses

After successful extraction of highly pure cpDNA from *A. littoralis*, we further applied the NLS method to extract chloroplasts and cpDNAs from four different species within the Poaceae: *Oryza sativa* L., *Zea mays* L., *Triticum aestivum* L., and *Dracaena sanderiana* Engl. (Appendix 2, protocol 5). The results showed that for

TABLE 2. Comparisons of quantity and quality of chloroplasts and cpDNAs obtained from different chloroplast extraction methods.

Method	Intactness ^a			cpDNA spectrophotometer analysis		
	Yield ^b (mg/mL)	Ferricyanide reduction (%)	ADP-photophosphorylation ($\mu\text{M ADP mg}^{-1}$ chlorophyll h^{-1})	DNA (ng/ μL)	OD 260/280 ^c	OD 260/230 ^d
Total DNA	—	—	—	2130	1.8	1.7
High salt	0.7	49	488	890	1.6	1.9
Modified high salt	2.7	61.2	228.2	1437	2.2	2.5
NaOH low salt	2.4	85.1	113.1	342	1.8	1.2

Note: — = value not measured in this sample.

^aChloroplast intactness measured by ferricyanide reduction and ADP-photophosphorylation assays.

^bAbsorption at 625 nm wavelength.

^cRatio of light absorbance at 260 and 280 nm.

^dRatio of light absorbance at 260 and 230 nm.

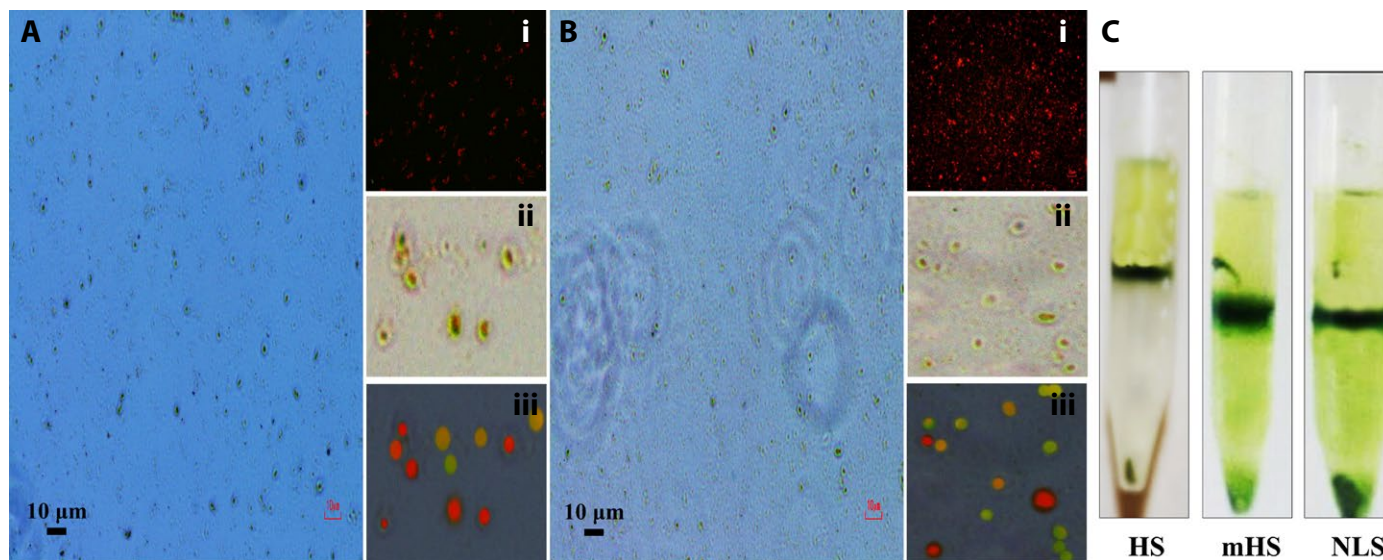


FIGURE 4. Brightfield and fluorescent microscopy images of isolated chloroplasts using the modified high-salt isolation method (A) and the NaOH low-salt isolation method (B). Results of chlorophyll fluorescence analysis are shown for each method: (i) chlorophyll red autofluorescence; (ii) phase contrast, zoom-in views; (iii) chlorophyll fluorescence analysis after CFDA staining. (C) Chloroplast fractions obtained from each isolation method after centrifuging on a Percoll density gradient (40%).

each species, the NLS method yielded a sufficient amount of chloroplast fraction according to total chlorophyll content (*O. sativa*: 2.8 mg/mL, *Z. mays*: 1.8 mg/mL, *T. aestivum*: 1.7 mg/mL, and *D. sanderiana*: 1.9 mg/mL). The chloroplast integrity was also high, calculated as 92%, 82%, 76.5%, and 85% for *O. sativa*, *Z. mays*, *T. aestivum*, and *D. sanderiana*, respectively. Furthermore, gel electrophoresis (Appendix S2B) and absorbance measurement at 230, 260, and 280 nm confirmed that isolated cpDNAs were of good quality. The cpDNA concentrations were calculated to be 192, 290, 485, and 262 ng/ μ L for *O. sativa*, *Z. mays*, *T. aestivum*, and *D. sanderiana*, respectively. Altogether, these results suggest that the NLS method can be successfully used to extract chloroplasts and cpDNAs from these species, and it is expected to be useful for intact chloroplast extraction and highly pure cpDNA preparation from other species.

Chloroplast whole genome reads analysis of *A. littoralis*

To estimate the purity level of isolated cpDNA from *A. littoralis* with the new NLS isolation protocol and to test the efficiency of genome assembly, we used the HiSeq 2000 Illumina sequencing platform (Illumina, San Diego, California, USA) to obtain whole genome sequence data (Appendix 2, protocol 6). With 2 μ g of isolated cpDNA, a total of 8,745,956 reads assigned to 13.1 Mbp of sequence data were generated. Reference-guided genome mapping

using chloroplast and mitochondrial genomes of different species (Appendices S3, S4) showed that the average aligned reads for cpDNA and mtDNA among the analyzed species were about 44.3% and 0.81%, respectively. In this regard, *Astrebla pectinata* (Lindl.) F. Muell. ex Benth. was assigned as a chloroplast reference genome for the *A. littoralis* cpDNA assembly. In total, 99.6% of the reference genome was covered by *A. littoralis* raw reads, and no large gaps were found in the consensus sequence (Table 3). Moreover, a very high sequencing depth for the *A. littoralis* chloroplast genome (4405 \times) was obtained in comparison with the mitochondrial genome (about 2 \times) using *Sorghum Moench* as a mitochondrial reference (Table 3). We also aligned *A. littoralis* total reads against the target genes (*gigantea*, *cox1*, and *rbcl*) to compute the sequencing depths (Table 3). Based on the results, the sequencing depths of the target genes are consistent with the copy folds, suggesting that our chloroplast isolation method is reliable and yielded a highly pure DNA sample for sequencing the plastid genomes (Figs. 2, 3; Table 3). Using the NLS method, the sequencing depth of the mitochondrial and nuclear genome is about 2 \times , which is far lower than the sequencing depths of the plastid genome (more than 4000 \times , Table 3); therefore, the effect of foreign reads can be relinquished when performing de novo assembly of the chloroplast genome. Correspondingly, these clean data proved effective for simplifying the assembling procedure by improving the percent of aligned reads and coverage index.

TABLE 3. Depth of coverage estimation of *Aeluropus littoralis* cpDNA aligned reads based on the reference genomes and target genes.

Gene/Genome	Chloroplast reference genome ^a	Mitochondrial reference genome ^b	Target genes		
			<i>cox1</i>	<i>gigantea</i>	<i>rbcl</i>
No. of total reads	8,745,956	8,745,956	8,745,956	8,745,956	8,745,956
No. of mapped reads	3,986,432	4538	1729	6	52,042
Mapped bases (bp)	597,964,854	680,701	208,768	840	6,552,714
Reference length (bp)	135,737	468,628	420	618	1412
Sequencing depth (\times)	4405	1	497	2	4640

^a*Astrebla pectinata*.

^b*Sorghum bicolor*.

CONCLUSIONS

It is important to note that, in our study, the quality of cpDNA was significantly influenced by the yield and intactness of isolated chloroplasts. Intact functional chloroplasts are a prerequisite for many fields of study, including photosynthesis, chloroplast metabolism, transport processes, proteomics, and photochemical analysis (Seigneurin-Berny et al., 2008; Kley et al., 2010; Holá et al., 2012; Paila et al., 2015). Therefore, the main goal of our study was to develop an optimal protocol that simultaneously generates an adequate amount of intact chloroplasts and highly pure cpDNA. Our results demonstrated that the HS and mHS methods (Bookjans et al., 1984; Shi et al., 2012) produced a highly damaged chloroplast fraction (Fig. 4) and yielded a cpDNA that was extremely contaminated with nuclear and mitochondrial DNAs (Figs. 2, 3B). We therefore modified the classical HS and mHS methods by including new steps (Fig. 1) and altering the buffer compositions (Table 1) to develop a new procedure (NLS method) for chloroplast extraction.

The new NLS method presented here improves chloroplast intactness by up to 80% and purity of cpDNA by up to 2.5-fold in comparison with high-salt methods (i.e., HS, mHS). In regard to cpDNA purity, we conclude that our modifications, including osmotic pressure adjustment (reducing ionic strength), a mild disruption process using ultrasonic waves after weakening cell wall structures with alkaline pretreatment and use of stabilizing agents in buffer components, efficiently protect the integrity of all the organelles. Thus, as a result, damaged nuclei and mitochondria were reduced in the solution, and the efficiency of the removal step via centrifuging was significantly increased. Finally, the pure cpDNA that was obtained in this study is sufficient for chloroplast whole-genome sequencing, and when sequenced on an Illumina platform led to enriched chloroplast reads. Based on these results, the approach presented here is expected to be useful for intact chloroplast isolation and highly pure cpDNA preparation from other species, especially grasses, using minor modifications such as adjusting the sample weight or buffer volume, lysing under milder or harder alkaline conditions, and using optimized sonication and centrifuging programs.

ACKNOWLEDGMENTS

The authors thank Dr. Ehsan Shokri for his invaluable discussion and advice during the course of this investigation. This research was supported by the Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT), University of Agricultural Sciences and Natural Resources (SANRU), Sari, Iran.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Primer sequences used in this study for qPCR analysis.

APPENDIX S2. (A) Total DNA (1) isolated from *Aeluropus littoralis* leaves according to the Dellaporta method (1-kbp DNA ladder; SM0311, Fermentas International Inc., Burlington, Ontario,

Canada). (B) Gel electrophoresis of cpDNA isolated from *Triticum aestivum* (1), *Dracaena sanderiana* (2), *Zea mays* (3), and *Oryza sativa* (4) (DNA ladder mix, ready-to-use; SM0333, Fermentas International Inc.).

APPENDIX S3. Mapping results of *Aeluropus littoralis* quality-filtered reads against the 19 chloroplast genomes of Chloridoideae.

APPENDIX S4. Mapping results of *Aeluropus littoralis* quality-filtered reads against all Poaceae mitochondrial genomes existing in the National Center for Biotechnology Information GenBank database.

LITERATURE CITED

- Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology* 24: 1–3.
- Bookjans, G., B. M. Stummann, and K. W. Henningsen. 1984. Preparation of chloroplast DNA from pea plastids isolated in a medium of high ionic strength. *Analytical Biochemistry* 141: 244–247.
- Dellaporta, S. L., J. Wood, and J. B. Hicks. 1983. A plant DNA miniprep: Version II. *Plant Molecular Biology Reporter* 1: 19–21.
- Diekmann, K., T. R. Hodkinson, E. Fricke, and S. Barth. 2008. An optimized chloroplast DNA extraction protocol for grasses (Poaceae) proves suitable for whole plastid genome sequencing and SNP detection. *PLoS ONE* 3: e2813.
- Do, H. D. K., J. S. Kim, and J. H. Kim. 2013. Comparative genomics of four Liliales families inferred from the complete chloroplast genome sequence of *Veratrum patulum* O. Loes. (Melanthiaceae). *Gene* 530: 229–235.
- Heber, U., and K. Santarius. 1970. Direct and indirect transfer of ATP and ADP across the chloroplast envelope. *Zeitschrift für Naturforschung B* 25: 718–728.
- Holá, D., M. Kočová, O. Rothová, E. Hlízová, L. Fridrichová, Z. Lhotáková, and J. Albrechtová. 2012. A universal method for the isolation of photochemically active broken chloroplasts from conifer needles and its possible application in photosynthetic studies. *Photosynthetica* 50: 291–304.
- Islam, M. S., B. Studer, S. L. Byrne, J. D. Farrell, F. Panitz, C. Bendixen, I. M. Møller, and T. Asp. 2013. The genome and transcriptome of perennial ryegrass mitochondria. *BMC Genomics* 14: 202.
- Jansen, R. K., L. A. Raubeson, J. L. Boore, T. W. Chumley, R. C. Haberle, S. K. Wyman, A. J. Alverson, et al. 2005. Methods for obtaining and analyzing whole chloroplast genome sequences. *Methods in Enzymology* 395: 348–384.
- Kingsford, C., M. C. Schatz, and M. Pop. 2010. Assembly complexity of prokaryotic genomes using short reads. *BMC Bioinformatics* 11: 21.
- Kley, J., M. Heil, A. Muck, A. Svatoš, and W. Boland. 2010. Isolating intact chloroplasts from small *Arabidopsis* samples for proteomic studies. *Analytical Biochemistry* 398: 198–202.
- Lang, B. F., and G. Burger. 2007. Purification of mitochondrial and plastid DNA. *Nature Protocols* 2: 652–660.
- Langmead, B., and S. L. Salzberg. 2012. Fast gapped-read alignment with Bowtie 2. *Nature Methods* 9: 357.
- Lilley, R. M., and D. A. Walker. 1973. The measurement of cyclic photophosphorylation in isolated chloroplasts by determination of hydrogen ion consumption. An evaluation of the method using titration at constant pH. *Biochimica et Biophysica Acta, Bioenergetics* 314: 354–359.
- Lutz, K. A., W. Wang, A. Zdepski, and T. P. Michael. 2011. Isolation and analysis of high quality nuclear DNA with reduced organellar DNA for plant genome sequencing and resequencing. *BMC Biotechnology* 11: 54.
- Mullet, J. E., and N. H. Chua. 1983. In vitro reconstitution of synthesis, uptake, and assembly of cytoplasmically synthesized chloroplast proteins. *Methods in Enzymology* 97: 502–509.
- Nasiri, N., E. Shokri, and G. A. Nematzadeh. 2012. *Aeluropus littoralis* NaCl-induced vacuolar H⁺-ATPase subunit c: Molecular cloning and expression analysis. *Russian Journal of Genetics* 48: 1199–1206.

- Paila, Y. D., L. G. Richardson, and D. J. Schnell. 2015. New insights into the mechanism of chloroplast protein import and its integration with protein quality control, organelle biogenesis and development. *Journal of Molecular Biology* 427: 1038–1060.
- Pongratz, P., and E. Beck. 1978. Diurnal oscillation of amylolytic activity in spinach chloroplasts. *Plant Physiology* 62: 687–689.
- Santarius, K. A. 1973. The protective effect of sugars on chloroplast membranes during temperature and water stress and its relationship to frost, desiccation and heat resistance. *Planta* 113: 105–114.
- Schulz, A., J. Knoetzel, H. V. Scheller, and A. Mant. 2004. Uptake of a fluorescent dye as a swift and simple indicator of organelle intactness: Import-competent chloroplasts from soil-grown *Arabidopsis*. *Journal of Histochemistry and Cytochemistry* 52: 701–704.
- Seigneurin-Berny, D., D. Salvi, A. J. Dorne, J. Joyard, and N. Rolland. 2008. Percoll-purified and photosynthetically active chloroplasts from *Arabidopsis thaliana* leaves. *Plant Physiology and Biochemistry* 46: 951–955.
- Shi, C., N. Hu, H. Huang, J. Gao, Y. J. Zhao, and L. Z. Gao. 2012. An improved chloroplast DNA extraction procedure for whole plastid genome sequencing. *PLoS ONE* 7: e31468.
- Takabe, T., M. Nishimura, and T. Akazawa. 1979. Isolation of intact chloroplasts from spinach leaf by centrifugation in gradients of the modified silica "Percoll". *Agricultural and Biological Chemistry* 43: 2137–2142.

APPENDIX 1. Voucher information for species used in this study.

Species	Voucher specimen accession no. ^a	Collection locality ^b	N
<i>Aeluropus litoralis</i> (Gouan) Parl.	MMI-001-NN	Mazandaran, Miankaleh	20
<i>Oryza sativa</i> L.	MSI-002-NN	Mazandaran, Sari	6
<i>Zea mays</i> L.	MSI-003-NN	Mazandaran, Sari	6
<i>Triticum aestivum</i> L.	KRM-004-NN	Khorasan razavi, Mashhad	6
<i>Dracaena sanderiana</i> Engl.	MSI-005-NN	Mazandaran, Sari	3

Note: N = number of individuals; NN = Najmeh Nasiri, collector.

^aVouchers deposited at the Germplasm Bank, Genetic and Agricultural Biotechnology Institute of Tabarestan, University of Agricultural Sciences and Natural Resources, Sari, Iran.

^bLocality and Iranian province.

APPENDIX 2. Details of other laboratory protocols used in this study.

Protocol 1: For chloroplast DNA isolation, 8 mL of extraction buffer (20% SDS, 20 μ L of β -Me, 30 μ L of Proteinase K [10 mg/mL]) was added to the purified chloroplast pellet and incubated at 55°C for at least 3 h. Then, 1.5 mL of 5 M KAc (pH 5.2) was added to the lysed chloroplasts, and the centrifuge bottles were kept on ice for 30 min. Centrifugation was done at 10,000 \times g for 15 min, and the pellet was discarded. The supernatant was extracted with an equal volume of saturated phenol and chloroform/isoamyl alcohol (24 : 1) and centrifuged at 10,000 \times g for 20 min. Next, an equal volume of isopropyl alcohol was added to the upper clear aqueous phase, and the bottles were kept in the freezer (–20°C) overnight. Finally, the aqueous phase was centrifuged at 10,000 \times g for 20 min,

and the cpDNA pellet was washed with 70% ethanol, air-dried, and redissolved in TE buffer (Shi et al., 2012).

Protocol 2: The yield of isolated chloroplasts was estimated based on the milligrams of chlorophyll. For this, 10 μ L of the chloroplast suspension was added to 1 mL of an 80% acetone solution and was mixed well. After a few minutes, the mixture was centrifuged (3000 \times g, 2 min), and the supernatant was retained. Then, the absorbance of the supernatant at 652 nm was measured. The absorbance of the chlorophyll was multiplied by the dilution factor (100) and was divided by the extinction coefficient of 36 to obtain the milligrams of chlorophyll per milliliter of the chloroplast suspension (Arnon, 1949).

Protocol 3: For the phosphorylation assay, isolated chloroplasts from the modified high-salt (mHS) and NaOH low-salt (NLS) preparations were added to the isotonic medium and distilled water in two separate reaction containers. The water-containing vessel was stirred for 1 min and then double-strength reaction medium was added to restore isotonic conditions following the osmotic shock. The rates of cyclic photophosphorylation by the chloroplasts were then measured simultaneously and compared between the two vessels.

Protocol 4: Chloroplasts were equilibrated for 5 min with an equal volume of carboxyfluorescein diacetate (CFDA) at a final concentration of 0.0025% w/v under gentle shaking. Then, 10 μ L of the stained chloroplasts was placed on a slide and analyzed using a Nikon Eclipse Ni-E microscope (Nikon Corporation, Tokyo, Japan) equipped with FITC filters (Schulz et al., 2004).

Protocol 5: Fresh leaves (15 g) of *Oryza sativa*, *Zea mays*, *Triticum aestivum*, and *Dracaena sanderiana* were extracted according to the NLS method as described above. The cpDNA was obtained from the isolated chloroplast fraction and purity was measured by ultraviolet-visible spectroscopy. Chloroplast yield and integrity were assessed by chlorophyll content and photoreduction assay, respectively.

Protocol 6: Approximately 2 μ g of cpDNA was sequenced as paired-end using the Illumina HiSeq 2500 platform (Illumina, San Diego, California, USA) at the Beijing Genomics Institute (BGI). To identify the best chloroplast and mitochondrial reference genomes based on the coverage level parameter, all quality-filtered reads were mapped against the 19 chloroplast genomes of Chloridoideae and all Poaceae mitochondrial genomes (Appendices S3, S4), respectively, using Bowtie 2.0 (Langmead and Salzberg, 2012). Finally, the accurate sequencing depths of the mitochondrial and plastid genomes based on the selected reference genomes and target genes (*gigantea*, *cox1*, *rbcl*) were calculated to further assess the cpDNA purity.