An efficient method of isolating mitochondrial and chloroplast DNA from higher plants

Weiqi Li*, D. Z. Skinner*,† and George H. Liang*

*Program of Genetics, Department of Agronomy, Throckmorton Hall, Kansas State University, KS 66506-5501, USA
†USDA-ARS, Kansas State University, Manhattan, KS 66506-5501, USA

Simultaneously isolating chloroplast and mitochondrial DNA was made from higher plants (monocotyledonous or dicotyledonous). The procedure is rapid, requiring about 6 h to complete; relatively inexpensive, requiring no ultracentrifugation or column chromatography, and broadly applicable. The DNA isolated is of high quality, readily cleaved with restriction enzymes, and efficiently cloned into plasmid vectors. This procedure should prove useful to researchers studying plant organellar DNA as a tool in evolutionary, taxonomic or genetic analysis.

A prerequisite to study plant organellar DNA is developing an efficient procedure to isolate chloroplast (cp) and mitochondrial (mt) DNA of high quality. The procedure should be applicable to many species, relatively inexpensive, rapid, with reasonable yields. One of the most important factors in judging the efficiency of such procedures is the quality of the DNA obtained. The organellar DNA so obtained must be relatively free from contaminating nuclear DNA and must be of sufficient purity so that it can be cleaved readily by restriction endonucleases and cloned.

Classical approaches to purifying cp- or mtDNA have been using a combination of DNase treatments on isolated organelles and multiple centrifugations, sometimes including a CsCl density gradient ultracentrifugation step. These approaches are time-consuming and expensive. It was reported that the successful isolation of cpDNA from pea was possible in a high ionic strength buffer without DNase treatment or density gradient ultracentrifugation, but with a column chromatograph step[1]. We have developed a procedure with which we can rapidly isolate both cp- and mtDNA from alfalfa (*Medicago sativa* L.), a dicotyledonous plant species; sorghum [*Sorghum bicolor* (L.) Moench] and wheat (*Triticum aestivum* L.), both monocotyledonous crop species. The procedure does not require column chromatography and can be completed in about 6 h.

Plants of alfalfa, sorghum and wheat were grown in greenhouses or growth chambers and leaves were harvested and incubated overnight in darkness at room temperature (22°C) in covered containers. Three grams of alfalfa leaves or 30 g of sorghum and wheat leaves were placed in 5 to 10 volumes of ice-cold homogenization buffer (50 mM Tris at pH 8, 25 mM EDTA, 10 mM mercaptoethanol, 0.1% egg albumin and 1.25 M NaCl) in a prechilled 1 l waring blender and homogenized at full speed with 3 to 5 sec bursts. The homogenate was squeezed gently through eight layers of cheesecloth and then filtered with Miracloth (Calbiochem). The filtrate was centrifuged at 1,5000 *g* for 5 min (Sorvall GSA rotor, 3,100 rpm). The supernatant containing mitochondria and a few residual chloroplasts was saved. The pellet containing chloroplasts was resuspended gently with a small camel's hair brush in about 50 ml homogenization buffer and again centrifuged at 1,500 *g* for 5 min. The pellet was resuspended in 6 ml resuspension buffer (25 mM Tris at pH 8, 10 mM EDTA). Lysis of the plastids was achieved by the addition of sodium dodecyl sulphate (SDS), sarkosyl, and proteinase K to final concentrations of 0.5%, 2% and 50 µg/ml respectively and incubated at 37°C for 1 h. After lysis, the preparation may be stored overnight at −20°C with no detectable loss of DNA. The lysate was then extracted twice with an equal volume of phenol : chloroform : isoamyl alcohol (24 : 24 : 1) saturated with 0.1 M Tris at pH 8 and twice with chloroform. The nucleic acid was precipitated with the addition of one volume of 2 M NH_4OAc and two volumes of ice-cold absolute ethanol. Th pellet was collected with centrifugation at 10,000 *g*, rinsed with 80% ethanol, dried at 37°C, and dissolved in 20 µl TE (10 mM Tris, 0.5 mM EDTA). The nucleic acid may be treated with RNase, if desired.

The supernatant from the first centrifugation was centrifuged again at 1,500 *g* to remove residual chloroplasts and the pellet was discarded. The supernatant was centrifuged at 10,000 *g* for 15 min to collect the mitochondria; the pellet was resuspended in about 50 ml homogenization buffer and centrifuged again at 10,000 *g* for 15 min. The pellet was resuspended in the resuspension buffer and isolation of mtDNA then proceeded as with cpDNA.

Restriction endonuclease digestions with *Eco*RI, *Bam*HI, *Hin*dIII, *Pst*I and *Hae*III were made according to the suppliers' recommendations. Agarose gel electrophoresis was carried out with 0.7% Seakem LE agarose (FMC) in 0.5 × TBE buffer[1] for 17 h at 3–5 V/cm. Gels were stained with ethidium bromide (0.5 g/ml, 20 min) and photographed using transmitted UV light.

The organellar DNA yields from 3 g of alfalfa leaves or 30 g of sorghum or wheat leaves was about 10 µg cpDNA and 6 µg mtDNA; estimated by band intensity comparisons to known amounts of λ and mtDNA that was not digested with restriction endonucleases as shown in Figure 1. The mtDNA preparations also contained fairly high-quality RNA (Figure 1), as demonstrated by the disappearance of heavy bands from the gel after soaking in RNase A solution. Restriction enzyme-digested cpDNA and mtDNA samples are shown in Figures 2

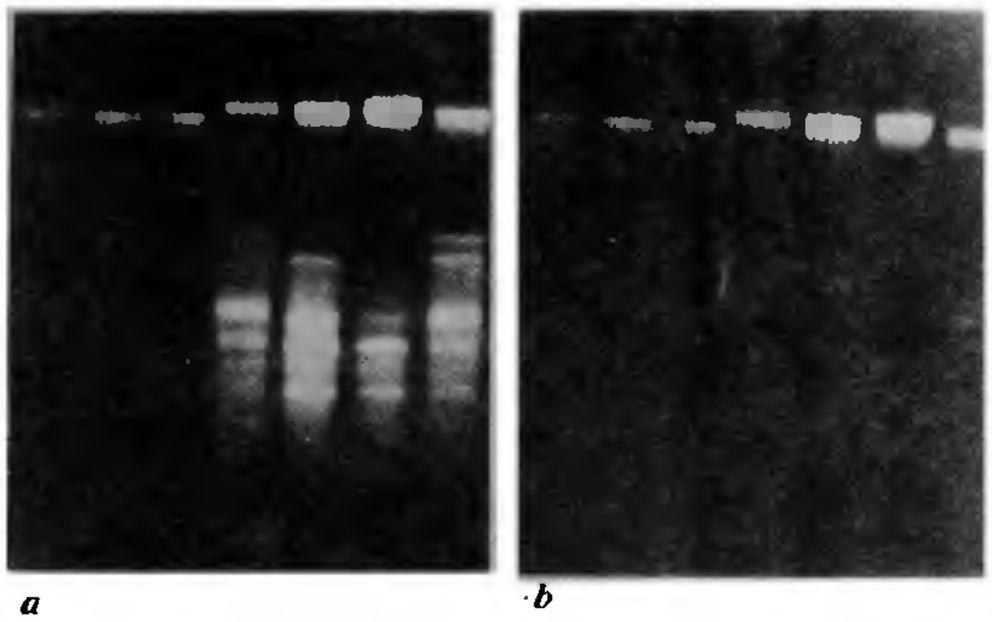


Figure 1. Purified alfalfa cpDNA (first three lanes) and mtDNA (last four lanes), before treatment with RNase A (*a*) and after soaking in RNase A (5 µg/ml) for 2 h at 37°C (*b*).

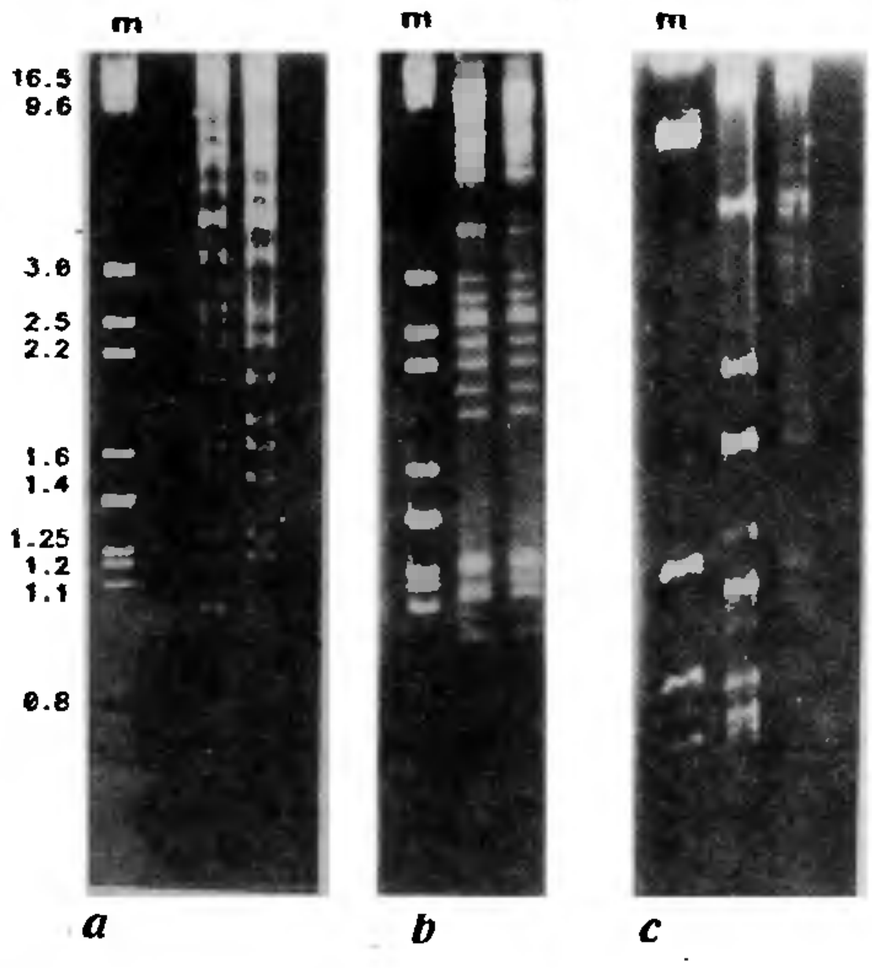


Figure 2. Alfalfa mtDNA digested with *Eco*RI (*a*), sorghum mtDNA digested with *Bam*HI (*b*), and wheat cpDNA and mtDNA digested with *Eco*RI (*c*). Lanes marked 'm' contain λDNA digested with *Bgl*I used as a size marker; fragment sizes are indicated in kilobase pairs.

and 3. The DNA cleaved completely with all enzymes used, resulting in very sharp bands. Little or no background smearing (indicative of contaminating nuclear DNA) was observed. This loss of nuclear DNA apparently results from the high salt concentration in the homogenization buffer[2]. Presumably, the high ionic strength results in a dissociation of the nuclear envelope and perhaps the chromosomes, providing a simple method of separating the plastids from nuclear DNA. We have generated several hundred plasmid clones of alfalfa mtDNA isolated with this procedure, indicating that the preparations are largely free from contaminating substances that interfere with ligation and cloning.

Molecular analysis of organellar genomes has been

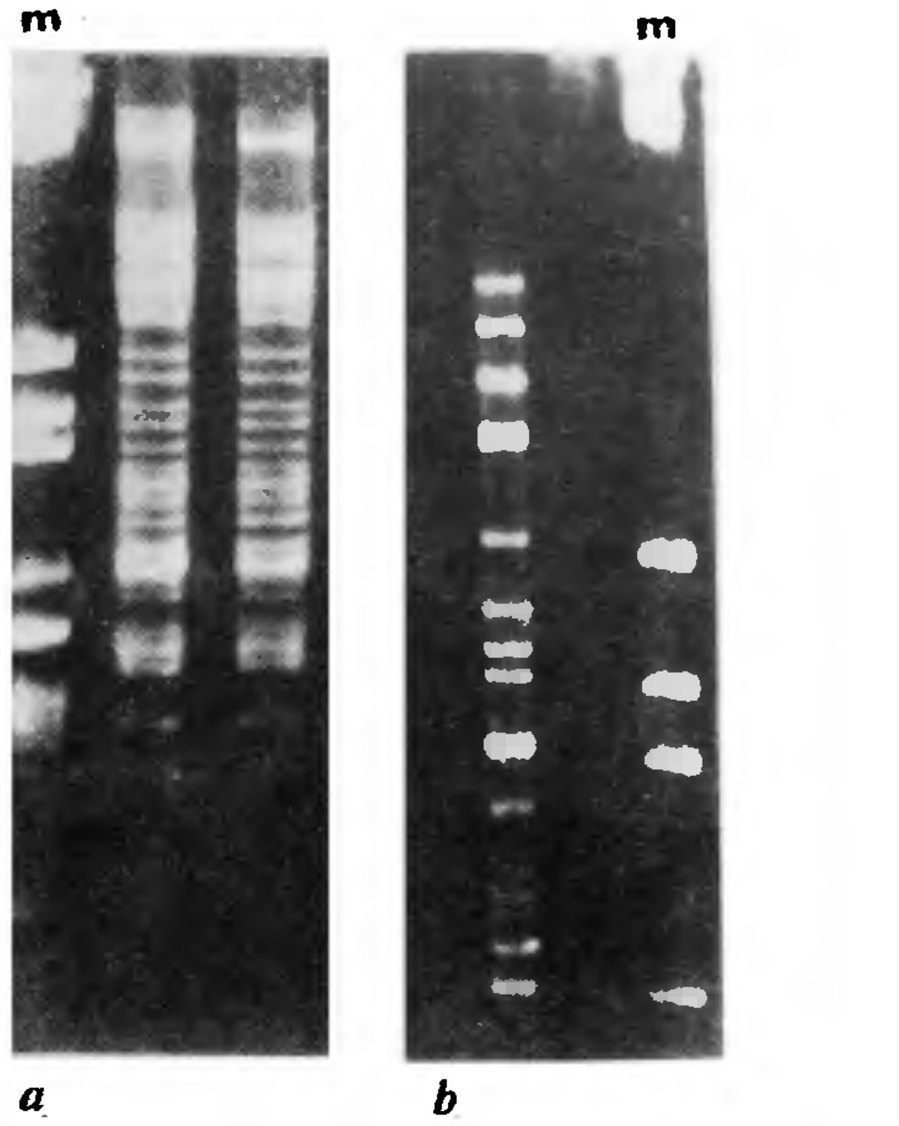


Figure 3. Alfalfa cpDNA digested with *Eco*RI (*a*) and sorghum cpDNA digested with *Hin*dIII (*b*), respectively. Lanes marked 'm' contain λDNA digested with *Bgl*I used as a size marker; fragment sizes are indicated in Figure 2.

widely used in plant phylogeny[3–6] and restriction site mapping[7–11]. The described procedure provides a rapid, inexpensive means of simultaneously isolating cpDNA and mtDNA from either monocotyledonous[12] or dicotyledonous plant species[13] and should facilitate molecular analysis of those organellar genomes. The procedure yields organellar DNA of high quality and purity without density gradient ultracentrifugation or column chromatography. The DNA obtained can be used for restriction enzyme analysis and cloning. Thus the procedure we introduced here should prove useful to researchers studying organellar DNA of many angiospermic plant species.

1. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, NY, 1989, 2nd edn.
2. Bookjans, G., Stummann, B. M. and Henningsen, K. W., *Anal. Biochem.*, 1989, **141**, 244–247.
3. Kellogg, E. A., *Am. J. Bot.*, 1992, **79**, 186–197.
4. Ogihara, Y. and Tsunewaki, K., *Theor. Appl. Genet.*, 1988, **76**, 321–332.
5. Palmer, J. D., Jansen, R. K., Michaels, H. J., Chase, M. W. and Manhart, J. R., *Ann. Missouri Bot. Garden*, 1988, **75**, 1180–1206.
6. Whitkus, R., Doebley, J. and Lee, M., *Genetics*, 1992, **132**, 1119–1130.
7. Davis, J. I. and Soreng, R. J., *Am. J. Bot.*, 1993, **80**, 1444–1454.
8. Downie, S. R. and Palmer, J. D., *Ann. Missouri Bot. Garden*, 1989, **79**, 266–283.
9. Duvall, M. R. and Doebley, J. F., *Syst. Bot.*, 1990, **15**, 472–480.

10. Jansen, R. K., Holsinger, K. E., Michaels, H. J. and Palmer, J. D., *Evolution*, 1991, **44**, 2089–2105.
11. Soreng, R. J., Davis, J. I and Doyle, J. J., *Plant Syst. Evol.*, 1990, **172**, 83–97.
12. Guo, J., Ph D thesis, Kansas State Univ, USA, 1993.
13. Saori, Oki, MS thesis, Kansas State Univ, USA, 1995.

ACKNOWLEDGEMENTS. This study is cooperative investigations of the US Department of Agronomy and Kansas Agricultural Experiment Station. WL was partially supported by a grant from the Kansas Grain Sorghum Commission and the Chemistry Institute, Academia Sinica.

Received 25 September 1995; accepted 22 November 1995

Antitumour activity of amooranin from *Amoora rohituka* stem bark

T. Rabi

Department of Shalya-Shalakya, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221 005, India

The *in vivo* antitumour activity of amooranin was determined using N-nitrosomethyl urea-induced Sprague–Dawley rats mammary adenocarcinoma. Intraperitoneal administration of the compound at a dose of 10 or 20 mg/kg/day prolonged the mean survival time of the animals and significantly reduced tumour sizes.

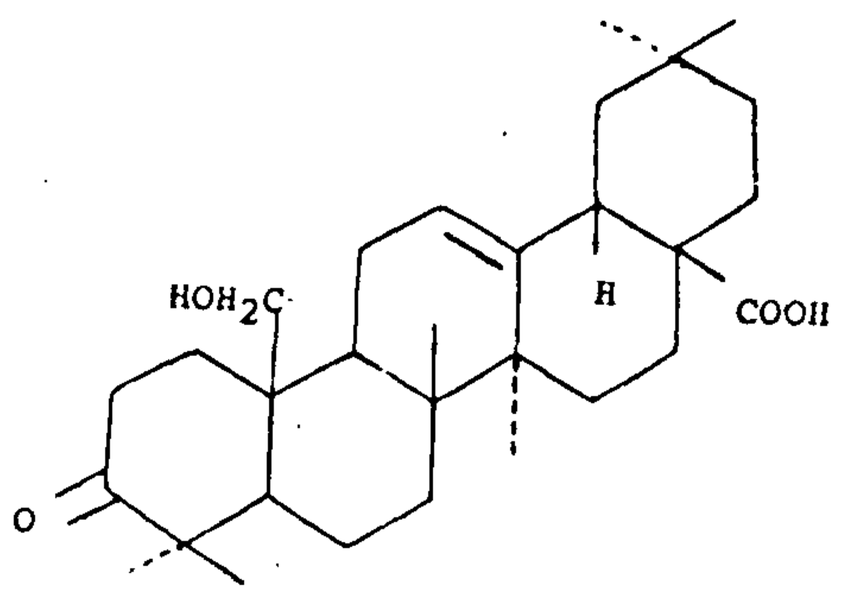
AMOORANIN is a triterpene acid with a novel skeleton isolated from *Amoora rohituka*, which is a medicinal plant used in the diseases of spleen, liver, tumours, abdomen and in rheumatism[1]. The stem bark was collected from Varanasi, India. The botanical identification of *Amoora rohituka* was confirmed by Suman, BHU, Varanasi, India (A voucher specimen is available in the herbarium).

The bark (2.1 kg) was washed, air-dried and ground into a coarse powder. It was extracted in a soxhlet with ethanol for 48 h. The ethanolic extract was vacuum evaporated to give a black residue (240 g). This residue was diluted with water and fractioned successively with petroleum ether, ethyl acetate and *n*-butanol to give petroleum ether, ethyl acetate, *n*-butanol and water-soluble fractions. The petroleum ether, ethyl acetate and *n*-butanol fractions were concentrated *in vacuo*, dried to constant weight at 37°C. The ethyl acetate extractive (50 g) was directly chromatographed on a silica gel column using ethyl acetate as eluant. The ethyl acetate fraction was chromatographed on a silica gel column, resulting in the isolation of a compound provisionally assigned as AR-II. It produced white crystal (200 mg) on crystallization from methanol.

White crystals, m.p. 276–280°C, IR (KBr) Vmax cm^{-1}, 3433 (OH), 2854 (C=O), 1689 (C=C). 1585, 1383, 1032; EIMS: M^+ 470, 440, 426, 409, 396, 379, 248, 223, 203, 191 and 133; ^{1}H-NMR (600 MHz $CDCl_3$): δ0.75 (3H, s, 26-Me), 0.91 (3H, s, 29-Me), 0.93 (3H, s, 30-Me), 0.98 (3H, s, 24-Me), 1.13 (3H, s, 23-Me), 1.25 (3H, s, 27-Me), 2.44 (2H, m, C2-H), 2.82 and 2.88 (1H, dd, C18-H), 3.46 and 3.96 (1H each, pair of AB doublets, CH_2OH), 5.3 (1H, t, C12–H).

A sample of AR-II (30 mg) treated with ethereal diazomethane yielded a methyl ester (20 mg). The methyl ester (20 mg) was treated with Ac_2O/pyridine (1 : 1) at room temperature overnight. Workup in the usual way afforded methyl ester acetate AR-IIa; amorphous powder (15 mg); m.p. 161–165°C; ^{1}H-NMR (400 MHz $CDCl_3$): δ0.75 (3H, s, 26-Me), 0.95 (3H, s, 29-Me), 0.93 (3H, s, 30-Me), 0.98 (3H, s, 24-Me), 1.12 (3H, s, 23-Me), 1.23 (3H, s, 27-Me), 2.0, (3H, s, O–C–CH_3), 3.6 (3H, s, COOMe), 3.9 and 4.6 (1H each, pair of AB doublets, CH_2OH), 5.26 (1H, t, C12–H).

Treatment of methyl ester of AR-II (13 mg) with sodium borohydride furnished a diol AR-IIb amorphous powder (10 mg); m.p. 200–203°C. ^{1}H-NMR (600 MHz $CDCl_3$): δ0.75 (3H, s, 26-Me), 0.91 (3H, s, 29-Me), 0.93 (3H, s, 30-Me), 0.98 (3H, s, 24-Me), 1.12 (3H, s, 23-Me), 1.23 (3H, s, 27-Me), 2.82 and 2.88 (1H, dd, C18–11), 3.35 and 4.15 (1H each, pair of AB doublets, CH_2OH), 3.42 (1H, m, C3–H), 3.62 (3H, s, COOME) and 5.26 (1H, t, C12–H).



For *in vivo* studies, N-nitrosomethyl urea-induced primary breast adenocarcinomas in Sprague–Dawley rats were transplanted sc beneath the lower thoracic mammary fat pads into female Sprague–Dawley rats 60–90 days old and divided into four groups of six animals each. One group was kept as control and the other three for treatment. On day 50 after tumour transplantation, and when the tumour volume was 50–150 mm^3, the treatment by intraperitoneal administration of amooranin or tamoxifen started in doses of 10 mg/kg, 20 mg/kg or 0.5 mg/kg once every day for three weeks. Tumours