A RAPID AND EFFICIENT METHOD FOR THE ISOLATION OF MITOCHONDRIAL DNA FROM SMALL AMOUNTS OF SOME FRUIT TREES

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Abstract

The plant mitochondrial genome is a source of potentially useful markers for studies of phylogeny and population genetics. The isolation of high quality mtDNA from plants containing a high content of polyphenolics has been a difficult problem because the presence of metabolites that interfere with DNA isolation procedures and downstream applications such as PCR and DNA restriction. The present study describes a rapid and easy protocol that provides mtDNA of sufficient purity from small amounts (milligrams to 1gram) leaves tissue from different species of fruit trees such as pear (*Pyrus betulaefolia*), apple (Anna), olive (Kalamata), mango (Sukkary), and banana (Grand Nain), containing a high content of polyphenolics. The purity of mtDNA was confirmed by PCR amplification of genomic, mitochondrial gene and RAPD profiles. The mitochondrial COXIII gene of 400 bp was amplified while the genomic β-actin gene was not amplified. The technique is fast, inexpensive, reproducible, and suitable for PCR-based markers.

Keywords: Mitochondrial DNA; Extraction Method; Molecular Markers; RAPD.

INTRODUCTION

Plants have two cytoplasmic genomes, chloroplast (cp) DNA and mitochondrial (mt) DNA, which are generally uniparentally inherited. They are therefore a source of potentially useful markers for evolutionary and ecological studies. Plant mitochondria are the main site of the ATP synthesis and play an important role in nitrogen assimilation, amino acids biosynthesis, plant development, productivity, fertility, and disease resistance (Millar *et al.,* 2007). Since the first reports on mitochondrial DNA (mtDNA) restriction patterns, which provided evidence for the heterogeneity of higher plant mitochondrial genomes and for mtDNA recombination induced by protoplast fusion (Belliard *et al.,* 1979), there has been increasing interest in mitochondrial genomes.

Mitochondria have been studied for cytoplasmic male sterility (Zhang *et al.,* 2012), abiotic stress tolerance (Amirsadeghi *et al.,* 2007), alternative energy pathways in plants (Mackenzie and McIntosh, 1999). Phylogenetic and evolutionary relationships, (Schuster & Brennicke, 1994). Furthermore, isolation of plant mitochondrial DNA for use in different PCR techniques is one of the most important and time-consuming steps (Ejaz *et al*., 2013). Such techniques include the Southern blot analysis, polymerase chain reaction amplifications (AFLP, RAPD, SSR).

Molecular analysis of plant cytoplasmic variability requires efficient preparation of mitochondrial DNA (mtDNA). However, isolating pure mitochondrial DNA is often difficult for many studies due to mitochondrial physiology. Plant mitochondria is about 1% of the total cellular content of the cell and about 150 - 500 mitochondria are present in each plant cell as compared to 1,000 - 2,000 per cell in animals (Karp, 2008). Furthermore, PCR-based markers are therefore difficult to obtain, despite the large size of plant mtDNA (200 to 2400 kb), compared to 14–42 kb for animals and 18–176 kb for fungi (Backert *et al.,* 1997). This is due to the presence, in higher plant mtDNA, of introns, intergenic sequences, duplicate sequences and sequences of plastid and nuclear origin (Marienfeld *et al.,* 1999).

Different strategies have been proposed to isolate intact mitochondria and mitochondrial DNA. (Pérez *et al.,* 1990; Keech *et al.,* 2005; Cui *et al*., 2009) but most of them are either time consuming or ineffective when applied to small amounts of material. Procedures to isolate pure mitochondrial DNA are often drafted on two basic methodologies involving Percoll and Sucrose density gradient centrifugation (Keech *et al.,* 2005; Cui *et al*., 2009; Ejaz *et al*., 2013). However, these methodologies often require an expensive laboratory setup which is not possible for many researchers in low-budget labs. Furthermore, to our knowledge, no protocol providing significant mtDNA enrichment is available when starting from as little as one gram of tissue, and although several methods exist for mtDNA extraction from various plant tissues, there is no simple method for obtaining large quantities of mtDNA from plants containing a high content of polyphenolics. Satisfactory mtDNA samples generally can be prepared from the young leaves of common crop plants; in contrast, tree leaves yield low amounts of mtDNA, which is frequently unrestrictable because DNA degradation is mediated by secondary plant products such as phenolic terpenoids which may bind to DNA after cell lysis .

The isolation of high quality mtDNA from fruit trees containing high polyphenolics was a difficult problem. The present paper describes a rapid and easy

protocol that provides mtDNA of sufficient purity from small amounts (milligrams to 1gram) leaves tissue from different species of fruit trees such as pear (*Pyrus betulaefolia*), apple (Anna), olive (Kalamata), mango (Sukkary), and banana (Grand Nain), containing a high content of polyphenolics. Our protocol includes the isolation of mitochondria in a high salt medium to destroy nuclei, which are then eliminated along with the plastids by differential centrifugation. The purity of mtDNA was confirmed by PCR amplification of mitochondrial COXIII gene. The technique is fast, inexpensive, reproducible, and suitable for PCR-based markers.

MATERIALS AND METHODS

The following procedure was tested on fresh young leaf samples of five different kinds of fruit trees , pear (*Pyrus betulaefolia*), apple (Anna), olive (Kalamata), mango (Sukkary), and banana (Grand Nain) , grown at the orchard of Horticultures Research Institute, Giza governorate, during the year 2016.

Mitochondrial DNA isolation

The crucial step is to isolate intact organelles and lyse the nuclei in a medium of high ionic strength. This step was adapted from a previous protocol for the preparation of chloroplasts (Bookjans *et al.,* 1984) and mitochondria DNA (Nunzia *et al*., 2001). Plastids are eliminated by low speed centrifugations. All steps must be carried out at 4°C either in the cold room or on ice. mtDNA was extracted following the protocol described by (Nunzia *et al.,* 2001), with some modifications. About 0.5 g (fresh weight) of plant tissues was ground to fine powder in liquid N in a mortar. Before the tissue thawed, homogenization Buffer [100 mM Tris-HCl (pH 8.0), 1.5 M NaCl, 25 mM EDTA (pH 8.0), 0.2% BSA, and 56 mM β-mercaptoethanol] was added, and centrifuged 4 times (1000 g for 10 min; 3000 g for 15 min and 10 min; and finally, 12,000 g for 15 min) to pellet the mitochondria. To eliminate the nuclear DNA, mitochondrial pellets were resuspended in cold DNase I buffer . Mitochondria were pelleted by centrifugation at 12,000 g for 15 min. Mitochondria were lysed in buffer containing 5% sodium dodecyl sulfate, 50 mM Tris-HCl, pH 8, 25 mM EDTA-HCl, pH 8. Proteinase K was added to a final concentration of 25µg/ml and incubated at 37°C for 60 min with gentle shaking to avoid mechanical breaking of DNA. After the addition of 0.1 volume of 2 M ammonium acetate, the nucleic acids were extracted with an equal

volume of TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) saturated phenol/chloroform (1:1). The supernatant was precipitated at -20°C for 1 h with 2 volumes cold 100% ethanol. The mtDNA was pelleted by centrifugation at 15,000 g for 15 min. The mtDNA was further washed twice with 70% ethanol and once with absolute ethanol and then air dried for 30 min. The mtDNA was suspended in TE buffer and stored at -20°C until further use. After this step, although RNA was still present, the DNA was suitable for restriction enzyme analyses or PCR. If necessary, RNA was removed from the samples by adding 20 mg/ mL DNase-free RNase enzyme (Fermentas) and incubation at 37°C for 1 h. Samples were analyzed using 1.5% agarose gel electrophoreses in a 1X TBE (10 mM Tris-base, pH 8; 2.75 g/L boric acid; 1 mM EDTA-HCl, pH 8) solution and visualized under a UV transilluminator after staining with 0.5 mg/mL ethidium bromide.

PCR amplification

The PCR amplification was performed using the mtDNA samples for the mitochondrial COXIII gene (NCBI accession No. X52539), and nuclear β-actin gene (NCBI accession No. AB181991). The primers used are shown in Table (1). PCRs were performed in 20µL volumes with 50 ng mtDNA, 6 µL ddH2O, 1 µL (forward and reverse) primer for each specific-gene (COXIII gene, and β-actin gene), and 10 µL master mix. Reactions were performed in Techni (TC-512) PCR using the following temperature profile: 94°C for 5 min followed by 5 cycles at 94°C for 1 min, 60°C for 30 s and 70°C for 1 min, 35 cycles for 50 s at 94°C, 56 to 62°C with a 1.5°C step increase in temperature with 7s incubation at each temperature, 50 s at 72°C, and a final extension at 72°C for 6 min. PCR products were separated by 1.5% (w/v) agarose gel electrophoresis and ethidium bromide staining. We performed mitochondria- specific RAPD analysis with mtDNA from five fruit trees species using 500 nM 10-mer primers (Operon Technologies, Alameda, USA) Table (1). The amplification was done with Ready Mix RED Taq with 0.4 µg of mtDNA template for 2 min at 94°C and 40 cycles of 2 min at 94°C, 2 min at 38°C, 1 min and 30 s at 72°C, and a final extension of 15 min at 72°C. The products were separated by 1.5% (w/v) agarose gel electrophoresis and ethidium bromide staining.

Table 1. Primer pair (forward and reverse) sequence of mitochondria, nuclear gene, and RAPD.

Primers	**Nucleotide sequence (5' to 3')**	**Type of primers**
COXIII- F	GTATGGTGGCGGGATGTTCT	Mitochondria
COXIII-R	TAGTGGAGGGTGCTTGGTAA	Mitochondria
β-actin-F	CATAAAGGAGAAGCTCGCTTAC	Nuclear
β-actin-R	TCTCTTTGCTCATGCGATCAG	Nuclear
OP-D5	TGAGCGGACA	RAPD

RESULT AND DISCUSSION

In this study, We have described a procedure for obtaining adequate mtDNA preparations from small amounts (milligrams to 1gram) of leaves tissue from different species of fruit trees such as pear (*Pyrus betulaefolia*), apple (Anna), olive (Kalamata), mango (Sukkary), and banana (Grand Nain), containing a high content of polyphenolics. Two critical factors were taken into account when comparing mtDNA extraction protocols, i.e. DNA yield and quality (Mullis *et al.,* 1992), based on the gel electrophoresis, UV spectrophotometer analyses and PCR amplification analyses, Figure (1 and 2).

However, isolation of quality DNA from mitochondria is very difficult because of the presence of genomic DNA, plastid DNA, RNA, polysaccharides, and other metabolites. Varma *et al*., (2007) reported that extraction of large amounts of high-value, high molecular weight DNA can be limited by the presence of large amounts of phenolic compounds, DNases, and organelle DNA. The key step of this protocol, as previously described for cpDNA (Bookjans *et al.,* 1984), is the use of a high ionic strength medium to isolate mitochondria. Acidity, phenolic compounds and oxidation products that lead to the rapid inactivation of mitochondria were overcome by adding alkaline buffers, and BSA to the extract medium. Once isolated, the mitochondria were lysed and proteinase K was used to eliminate proteins instead of potassium acetate precipitation as described in Pérez *et al.,* (1990). To evaluate mtDNA purity, we investigated samples by amplifying the mitochondrial COXIII, and nuclear β-actin gene, Table (1). The products were separated by 1.5% (w/v) agarose gel electrophoresis. The 400-bp mitochondrial COXIII gene was amplified, while the β-actin was not amplified, and thus the mtDNA was not contaminated, (Figure 2).

The spectrophotometer mean values at A260/A280 and A260/A230 ratios using this method were 1.8 and 2, respectively. According to Meyer (2003), a higher value

of A260/A280 ratio indicates RNA contamination, whereas a lower value indicates protein contamination. The A260/ A280 ratio is typically used to determine the purity of isolated DNA. This ratio for pure double stranded DNA is generally considered to be 1.8-1.9. The isolated (all of these) mtDNAs were used for RAPD–PCR analysis using 10-mer primers (Operon Tech. Inc., USA), primers successfully generated reproducible and polymorphic products. Figures (3) demonstrate the results of RAPD-PCR profiles of the studied genotypes using (OP-D5) primer. This mtDNA preparation method yielded a predominance of high molecular weight mtDNA reliably produced PCR amplification products >1000 bp in length. Considering all factors involved, this method appears to be the most efficient, reliable and labor-effective mtDNA isolation procedure from plants containing a high content of polyphenolics such as fruit trees.

Numerous mitochondrial DNA isolation protocols have been described in the literature (Ejaz *et al.,* 2013, and Hwang *et al.,* 2001), all of which aim to maximize the mitochondrial DNA yield, while minimizing the contamination of nuclear DNA. However, these methods requires a large amount of starting material, has moderate precision and may have low reproducibility, making them unsuitable for high through put applications. Furthermore, to our knowledge, there is no method for obtaining large quantities of mtDNA from plants containing a high content of polyphenolics. Karaca *et al.,* (2005) reported that the polysaccharides, proteins, phenolic compounds, tannins, and pigments interfere with several biological enzymes such as DNA polymerases, ligases, and restriction endonucleases. Varma *et al.,* (2007) reported that extraction of large amounts of high-value, high molecular weight DNA can be limited by the presence of large amounts of phenolic compounds, DNases, and organelle DNA. In the present study, we have developed a method for mitochondrial DNA isolation with an undetectable amount of nuclear contamination, high reproducibility, less starting material, lower cost, and suitable for PCR and restriction enzyme analysis, etc. Furthermore this procedure has the potential for the scientific community running low on expenses, thereby reducing cost, time, and processing of mitochondrial studies.

CONCLUSION

`It is concluded that the protocol described here will be beneficial for scientific communities by providing a cheap and robust mitochondrial DNA isolation protocol for potential applications.

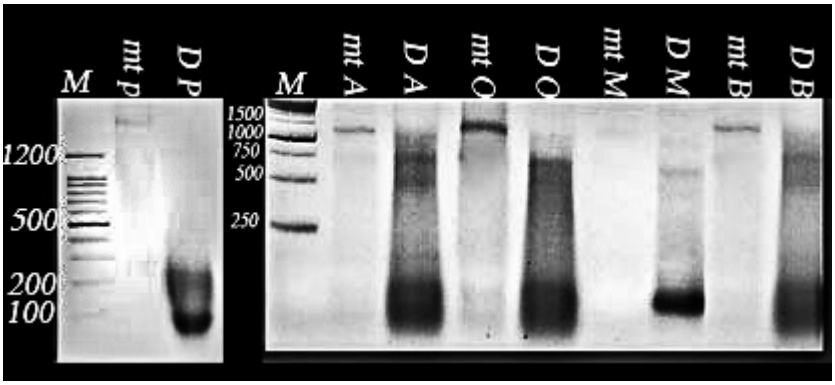

Figure 1. mtDNA and total DNA (mt and D) respectively, prepared from five different species of fruit trees. M: 1 kb DNA ladder, P: Pear (Pyrus betulaefolia), A: Apple (Anna), O: Olive (Kalamata), M: Mango (Sukkary),and B: Banana (Grand Nain).

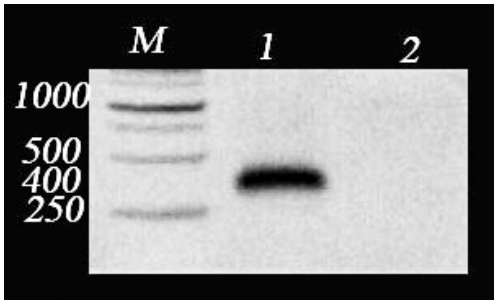

Figure 2. Agrose gel showing amplified COXIII gene, M: Marker; 1: COXII gene; 2: β-actin gene

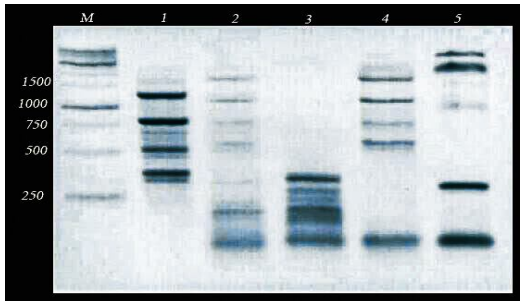

Figure 3. RAPD profiles of five different fruit tree species. mt DNAs were amplified using primer 5′-TGAGCGGACA-3′ (OP-D5). M: 1 kb DNA ladder. Lanes 1–5: 1, Pear (*Pyrus betulaefolia*); 2, Apple (Anna); 3, Olive (Kalamata)); 4 Mango (Sukkary),and 5, Banana (Grand Nain).

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طريقة سريعة وفعالة لعزل الحامض النووى لميتوكوندريا بعض اشجار الفاكهة بإستخدام كمية صغيرة من النسيج النباتى

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الجينوم الميتوكوندري النباتي هو مصدر من الواسمات الاصلية التي يحتمل أن تكون مفيدة لدراسة التطور والعشائر الوراثية. يمثل عزل الجينوم الميتوكوندرى من النباتات التي تحتوي على نسبة عالية من البوليفينول مشكلة صعبة نظرا للتداخل مع إجراءات عزل الحمض النووي والتطبيقات المستخدمة عن طريق تقنية سلسلة تفاعل البلمرة PCR. تصف هذه الدراسة بروتوكولا سريعا وسهلا يوفر ميتوكوندريا DNA ذات درجة نقاء كافيه من كميات صغيرة (ملليغرام إلى ١ غرام) من أنواع مختلفة من النسيج النباتى لبعض اشجار الفاكهة مثل الكمثرى (*Pyrus betulaefolia*) والتفاح (آنا) والزيتون (كلاماتا) والمانجو (سكرى.)، والموز (جراند نين)، التي تحتوي على نسبة عالية من البوليفينول. تم تأكيد نقاء ميتوكوندريا DNA عن طريق تضاعف الجين الخاص بميتوكوندريا DNA، وتحليل RAPD. تم تضاعف جين الميتوكوندريا COXIII في حين لم يتم تضاعف الجين الخاص بالجينوم β- actin مما يدل على نقاوة ميتوكوندريا DNA المستخلص. .هذه التقنية سريعة وغير مكلفة وقابلة للتكرار، ومناسبة لتطبيقات ال PCR.