

Chapter 2

DNA Extraction from Rice Endosperm (Including a Protocol for Extraction of DNA from Ancient Seed Samples)

Chiaki Mutou, Katsunori Tanaka, and Ryuji Ishikawa

Abstract

Deoxyribonucleic acid (DNA) extracted from endosperm can be effectively used for rapid genotyping using seed tissue, to evaluate seed quality from packaged grains and to determine the purity of milled grains. Methods outlined here are optimal procedures to isolate DNA from endosperm tissue of modern rice grains and of aged rice remains preserved between 50 and 100 years. The extracted DNA can be used to amplify regions of chloroplast genomic DNA (ctDNA), mitochondrial genomic DNA (mtDNA), and nuclear genomic DNA using standard PCR protocols. In addition, we describe an optimal procedure to process archaeological grain specimens, aged for a couple of thousand years, to isolate DNA from these ancient samples, referred to here as ancient DNA (aDNA). The aDNA can be successfully amplified by PCR using appropriate primer pairs designed specifically for aDNA amplification.

Key words Endosperm, Urea, Organellar DNA, nDNA, Ancient DNA

1 Introduction

Deoxyribonucleic acid (DNA) extracted from grain tissue of modern rice grains is frequently used for genotyping by polymerase chain reaction (PCR) in order to check the purity of seed sample lots. DNA can be extracted from endosperm tissue from aged grain material preserved between 50 and 100 years. However, extracting DNA from archaeological grain specimens can often be difficult and challenging. In addition, the PCR amplification of DNA from archaeological grain specimens, referred to here as ancient DNA (aDNA), is usually hampered due to low DNA content and inhibition by high starch and carbon in the samples. However, the removal of PCR inhibitors by appropriate filters and designing primers to amplify short genomic regions can be used as a strategy for the efficient application by PCR of desired targets in the aDNA [1, 2]. A robust and simple method for processing archaeological grain specimens to extract aDNA of good quality is necessary. Successful amplification of aDNA offers opportunities to examine

the genetics of modern-day crops resulting from evolution and human selection. In this chapter, we outline robust and simple DNA extraction methods which can be applied to rice grains derived from modern rice grains, aged rice grains preserved between 50 and 100 years, and archaeological grain specimens aged for a couple of thousand years.

2 Materials

2.1 For DNA Extraction from Modern Rice Grains

1. Dried rice kernels of Indica and Japonica varieties, CH55(6-11) and Nipponbare, respectively, were used as tissue for DNA extraction.
2. Mortar and pestle.
3. Urea solution: For 200 mL solution add the following: 84 g of urea, 14 mL of 5 M NaCl, 10 mL of 1 M Tris-HCl pH 8.0, 8 mL of 0.5 M EDTA pH 8, and 10 mL of 20 % Na-N-lauryl sarcosine. Add sterile distilled water to adjust the final volume to 200 mL. As urea is heat sensitive do not heat the solution above 70 °C and do not autoclave.
4. 5 M NaCl: For 1 L solution, take 292.2 g of sodium chloride and add 900 mL of distilled water. Mix to dissolve, and then adjust the final volume to 1 L with distilled water.
5. 1 M Tris-HCl, pH 8.0: For 1 L solution, take 121.1 g of Tris base and add 600 mL of distilled water. Adjust the pH to 8.0 by adding 28 mL of 0.1 N HCl and then dropwise till pH 8.0 is achieved. Adjust the final volume to 1 L with distilled water.
6. 0.5 M EDTA, pH 8.0: For 1 L solution, take 186.1 g of EDTA (disodium salt) and add 500 mL of distilled water. The EDTA will dissolve fully at pH 8.0. Adjust the pH to 8.0 as follows. Using a stirring bar to mix the solution, add 18 g of sodium hydroxide pellets followed by pH adjustment to 8.0 using dropwise addition of 10 M NaOH solution. Adjust the final volume to 1 L with distilled water.
7. TE-buffer, Tris-HCl pH 8.0 and 1 mM EDTA: To make 100 mL solution of TE buffer, take 1 mL of 1.0 M Tris-HCl (pH 8.0) and 0.2 mL EDTA (0.5 M) and adjust the final volume to 100 mL with distilled water.
8. 20 % w/v Na-N-lauryl sarcosine, in distilled water: To make 100 mL solution, take 20 g of Na-N-lauryl sarcosine and add 60 mL of distilled water. Gently stir for dissolving, but do not apply heat. Adjust the final volume to 100 mL with distilled water.
9. Sterile distilled water up to 400 mL.

10. Phenol/chloroform 1:1 v/v.
11. Isopropanol.
12. Microfuge, temperature controlled to 4 °C (tabletop centrifuge).

2.2 For DNA Extraction from Aged Rice Grains

For DNA extraction from dehulled rice grains preserved for about 100 years as stockpiled rice:

1. 2 mL screw-capped tubes.
2. Liquid nitrogen (liq N₂).
3. Bead grinder, example Multi-Beads Shocker (Yasui Kikai Co., Osaka, Japan).
4. CTAB solution: CTAB 8 g, 2 M Tris-HCl, pH 8, 10 mL, 0.5 M EDTA 8 mL, NaCl 16.36 g, PVP 2 g. Adjust the final volume to 200 mL with distilled water.
5. Extraction buffer (freshly prepared): Take 1 mL 2× CTAB solution and 2 µL 2-mercaptoethanol.
6. Water bath, 60 °C.
7. Chloroform/isoamyl alcohol (v/v, 24:1).
8. Microfuge (tabletop centrifuge).
9. 3 M Na-acetate: To prepare 100 mL of solution, take 40.82 g of sodium acetate trihydrate and add 50 mL of distilled water. Adjust the pH to 5.2 with glacial acetic acid, and then adjust the final volume to 100 mL with distilled water.
10. 70 % Ethanol, v/v in distilled water.
11. Isopropanol.
12. TE buffer, Tris-HCl pH 8.0 and 1 mM EDTA: To make 100 mL solution of TE buffer, take 1 mL of 1.0 M Tris-HCl (pH 8.0) and 0.2 mL EDTA (0.5 M) and adjust the final volume to 100 mL with distilled water.
13. TE + RNase solution: Take 10 mg RNase and dissolve in 1 mL of TE buffer.

2.3 For DNA Extraction from Archaeological Grain Specimens

1. Ancient rice remains such as chaff and grain.
2. Gamma ray-sterile distilled H₂O (γ-sterile distilled water).
3. Bead grinder, for example Multi-Beads Shocker (Yasui Kikai Co., Osaka, Japan).
4. 0.5 N NaOH: For 100 mL, take 2 g of NaOH and dissolve in 100 mL of distilled water.
5. Extraction buffer, 0.1 M Tris-HCl, pH 8.0: For 100 mL solution, take 10 mL of 1 M Tris-HCl, pH 8.0 (*see* Subheading 2.1, item 5) and add 90 mL of distilled water.
6. Water bath, 65 °C.

7. Microfuge (tabletop centrifuge).
8. 99.9 % absolute ethanol and cooled to or below -20°C .
9. 3 M Sodium acetate: To prepare 100 mL of solution, take 40.82 g of sodium acetate trihydrate and add 50 mL of distilled water. Adjust the pH to 5.2 with glacial acetic acid, and then adjust the final volume to 100 mL with distilled water.
10. Ethachinmate (Nippongene Co. Ltd, Japan).
11. 70 % Ethanol (v/v) in distilled water and cooled to or below -20°C .
12. 1 M Tris-HCl, pH 8.0: For 1 L solution, take 121.1 g of Tris base and add 600 mL of distilled water. Adjust the pH to 8.0 by adding 28.0 mL of 0.1 N HCl and then dropwise till pH 8.0 is achieved. Adjust the final volume to 1 L with distilled water.
13. 0.5 M EDTA, pH 8.0: For 1 L solution, take 186.1 g of EDTA (disodium salt) and add 500 mL of distilled water. The EDTA will dissolve fully at pH 8.0. Adjust the pH to 8.0 as follows. Using a stirring bar to mix the solution, add 18 g of sodium hydroxide pellets followed by pH adjustment to 8.0 using dropwise addition of 10 M NaOH solution. Adjust the final volume to 1 L with distilled water.
14. 10 mM TE buffer, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA: To make 100 mL solution of TE buffer, take 1 mL of 1.0 M Tris-HCl (pH 8.0) and 0.2 mL EDTA (0.5 M) and adjust the final volume to 100 mL with distilled water.
15. Column filter (Wizard Plus SV Minipreps DNA Purification Systems, Promega, USA). Ultraviolet light box emitting 254 nm ultraviolet light.

3 Method

3.1 For DNA Extraction from Modern Rice Grains (Urea Extraction with Phenol/Chloroform Treatment)

1. Use a mortar and pestle to crush each sample of rice kernel. One kernel per tube is enough to extract DNA for PCR protocols.
2. Transfer the powdered grain into an Eppendorf tube, and add 600 μL of urea solution
3. Mix the contents by vortexing a couple of times, and incubate the tubes for 1 h at room temperature (RT).
4. Centrifuge the tubes at $10,600\times g$ for 5 min at RT using a micro-centrifuge.
5. Transfer 300 μL of the supernatant into a clean Eppendorf tube, and add 200 μL of phenol/chloroform solution.
6. Mix the contents of the tubes by vortexing (*see Note 1*).

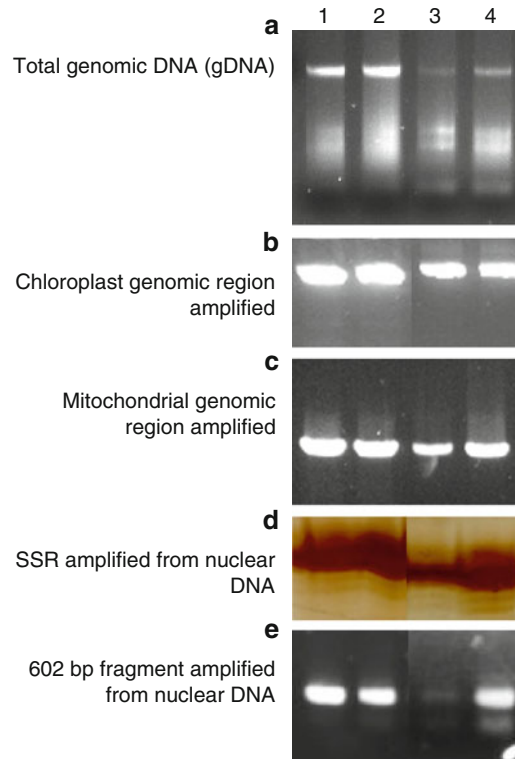


Fig. 1 Total genomic DNA (gDNA) isolated from leaf and grains of two rice varieties and successful amplification of chloroplast, mitochondrial, and nuclear regions by PCR. Lanes 1 and 3, *Oryza japonica* Nipponabare; lanes 2 and 4, *Oryza japonica* indica Ch55; lanes 1 and 2, leaf tissue; lanes 3 and 4, endosperm tissue. (a) Total genomic DNA resolved by agarose gel electrophoresis from rice grain, extracted with urea solution, and purified with phenol/chloroform treatment and from leaf tissue using an alternate protocol; (b) chloroplast genomic region amplified; (c) mitochondrial genomic region amplified; (d) an SSR motif amplified from nuclear DNA by a silver staining; (e) 660 bp fragment amplified from nuclear DNA

7. Centrifuge the tubes at 12,000 rpm for 5 min at 4 °C using a micro-centrifuge.
8. Transfer 200 μ L of the supernatant into a clean Eppendorf tube, and add equal volume of isopropanol.
9. Mix the contents gently by inverting the tubes, and incubate the tubes at 4 °C for 1 h.
10. Centrifuge the tubes at 12,000 rpm for 5 min at 4 °C using a micro-centrifuge.
11. Discard the supernatant, and dry the pellet under vacuum for 2 min.
12. Dissolve the pellet in 200 μ L of TE buffer, and then check the quality and quantity of DNA (*see Note 2*).
13. Dilute the DNA and then use in a PCR reaction (*see Note 3*, Fig. 1).

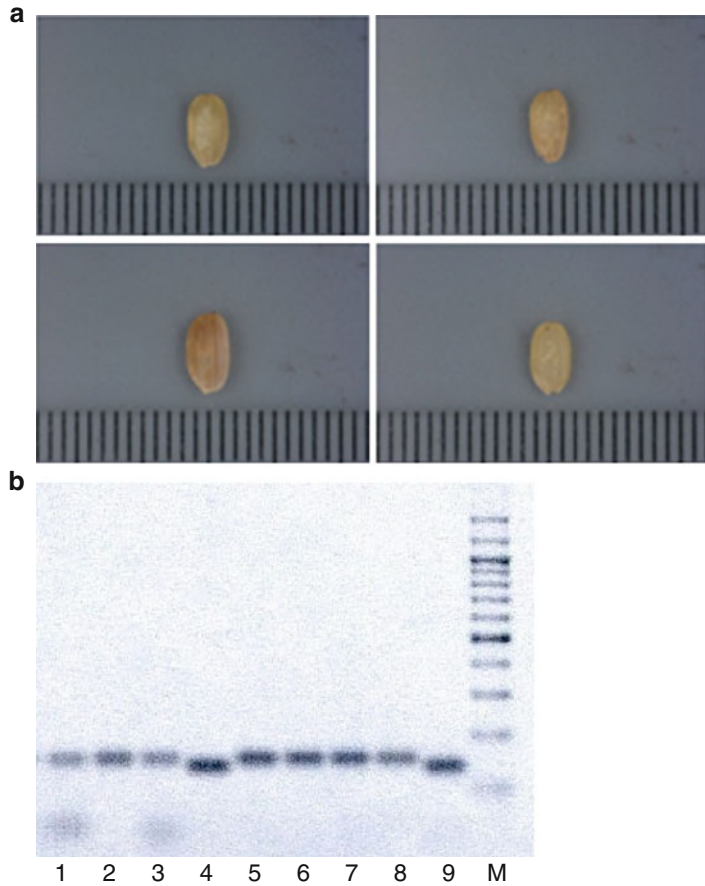


Fig. 2 Aged dehulled grains and their DNA successfully amplified. **(a)** Aged dehulled rice grain cultivated about 50–100 years ago used in this study; **(b)** successful PCR amplification of a short region from nuclear genomic DNA (nDNA) isolated from aged rice grains. PCR products amplified with the RM3604 SSR primers for rice. M, 100 bp DNA ladder; *lanes 1–9*, aged rice grain specimens

3.2 DNA Extraction from Aged Rice Grains

Dehull rice grains preserved for about 100 years as stockpiled rice; Fig. 2a.

1. Take grains of rice samples one each in 2 mL screw-capped tubes. Close the lid tightly, immerse the tube in liquid nitrogen for a few minutes to freeze the sample, and then grind the frozen rice grain into a fine powder using a bead grinder.
2. Before the sample thaws, add 600 μ L of extraction buffer and incubate the tubes in a 60 °C water bath for 30 min with periodic mixing by gently inverting the tubes every 10 min.
3. Add 600 μ L of chloroform/isoamyl alcohol (24:1), and mix by inverting the tubes several times until an emulsion is achieved.
4. Centrifuge the tubes at 12,000 rpm for 10 min using a microfuge.

5. Transfer about 500 μL of the supernatant into a clean sterile 2 mL Eppendorf tube, and add 300 μL of isopropanol and 50 μL of 3 M Na-acetate.
6. Mix the contents by inverting the tubes several times, and then incubate the tubes on ice for 30 min.
7. Centrifuge the tubes at 12,000 rpm for 10 min using a microfuge.
8. Discard the supernatant, and wash the pellet by adding 70 % ethanol followed by inverting the tubes a few times.
9. Centrifuge the tubes at 12,000 rpm for 5 min using a microfuge, and discard the supernatant.
10. Air-dry the pellet completely, add 30 μL of TE + RNase, and then incubate at 60 °C to dissolve the DNA.
11. Dilute the isolated DNA 1/20 in sterile TE buffer before using it for PCR amplification (*see* **Note 4**, Fig. 2b).

3.3 Extraction of DNA from Archaeological Grain Specimens

This method outlines a method to extract total DNA from archaeological grain specimens (aDNA) using a published procedure [3] but with minor modifications (*see* **Note 5**). Sterilize all pipet tips and tubes by exposing to ultraviolet light at 254 nm for 30 min. Ensure that all steps are carried out under clean bench conditions and wearing plastic gloves except when cleaning archaeological grain specimens and during the grinding procedure (*see* **Note 6**).

1. Clean each archaeological rice sample of any debris by sonication of samples under γ -sterile distilled water.
2. Dry each sample by air-drying and then using a bead grinder to grind the samples into a fine powder in the presence of 25 μL of 0.5 N NaOH.
3. Using a sterile spatula, transfer the ground sample each into a sterile Eppendorf tube.
4. Add 475 μL of extraction buffer, and gently mix by inverting the tubes several times.
5. Incubate the tubes in a 65 °C water bath for 10 min, and then centrifuge at 8,900 $\times g$ for 10 min using a microfuge.
6. Transfer the supernatant into a clean sterile Eppendorf tube containing 900 μL of cooled 99.9 % ethanol. Mix gently by inverting the tubes several times.
7. Centrifuge the tubes at 11,000 rpm for 10 min using a microfuge, and transfer the supernatant into a fresh sterile Eppendorf tube containing 30 μL of 3 M sodium acetate and 3.0 μL of ethachinamate. Mix gently by inverting the tubes several times.
8. Centrifuge the tubes at 11,000 rpm for 10 min using a microfuge, and transfer the supernatant into a fresh sterile

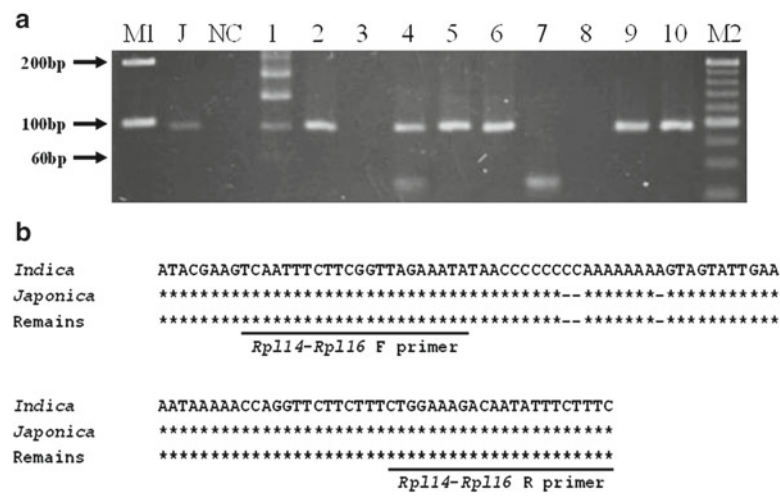


Fig. 3 Successful PCR amplification of a short region from nuclear genomic DNA (nDNA) isolated from archaeological rice grain specimens. **(a)** Electrophoresis of PCR products amplified, from ten archaeological rice grain specimens, with *Rp114-Rp116*-specific primer sets; M1, 100 bp DNA ladder; M2, 20 bp DNA ladder; J, Modern *japonica* cv. “Nipponbare”; NC, no-template negative control (dH₂O); 1–10, archaeological rice grain specimens; **(b)** remains, ancient rice remains such as chaff and grain (archaeological rice grain specimens). Sequence details of primers to amplify nuclear genomic regions corresponding to indica and japonica rice and archaeological rice specimens

- Eppendorf tube containing 300 μ L of 70.0 % ethanol. Mix gently by inverting the tubes several times.
9. Centrifuge the tubes at 11,000 rpm for 10 min using a microfuge.
 10. Discard the supernatant, and dry the pellet in air for a few minutes.
 11. Add 50 μ L of TE buffer, and incubate the tubes for 10–15 min in a 50 $^{\circ}$ C water bath to dissolve the pellet.
 12. After the pellet has dissolved, transfer the aDNA solution into a column filter to remove possible inhibitors affecting PCR.
 13. The column-filtered aDNA can be stored at 4 $^{\circ}$ C or at –22 $^{\circ}$ C for long-term storage.
 14. Use the extracted aDNA for PCR amplification (*see* **Note 7**, Fig. 3).

4 Notes

1. Make sure that an even emulsion is obtained.
2. This method was successfully used to isolate high-molecular-weight genomic DNA from endosperm tissue of Indica and Japonica varieties, CH55(6-11) and Nipponbare, respectively

(Fig. 1). Genomic DNA from leaf tissue, isolated using an alternate method (not described here), was used as a positive control. The DNA concentration was determined by spectrophotometry and also by intensity comparison of agarose gel-resolved high-molecular-weight standard DNA controls.

3. The extracted DNA can be diluted to 35 ng/μL and be used in a PCR for the successful amplification of chloroplast, mitochondrial, and genomic regions (Fig. 1b–c).
4. Amplified SSR amplicons were checked by electrophoresis (Fig. 2b).
5. In general, depending on materials such as plant or animal, large-scale or small-scale, and modern or ancient samples, a minor change might be needed in the DNA extraction procedures as well as in PCR condition and desired amplicon size.
6. In case of ancient DNA extraction, working condition should also be taken into account to avoid any contamination from outside sources of DNA. The use of a negative control in the PCR is essential, and we recommend undertaking extraction replicates.
7. PCR amplification is basically done by a commonly used PCR procedure. However, the PCR primer set should be designed to amplify PCR products with a size less than 100 bp. Due to deterioration of aDNA [4] during the PCR step leading to reduction of PCR amplification, we recommend a second PCR amplification step. This can be done using the PCR product from the first amplification as a template and the same primer pair or designing nested primer sets. Check the amplified PCR products by resolving in a 3.0 % agarose gel using electrophoresis.

References

1. Pääbo S, Higuchi RG, Wilson AC (1989) Ancient DNA and the polymerase chain reaction. *J Biol Chem* 264:9709–9712
2. Yang DY, Eng B, Wayne JS et al (1998) Technical note: improved DNA extraction from ancient bones using silica-based spin columns. *Am J Phys Anthropol* 105:539–543
3. Aoki C, Nishimura T, Yasui S, et al (1999) Modification of DNA and RNA extraction method in rice leaf using Multi Bead Shocker. *Breeding Res Suppl* 1:18
4. Pääbo S, Poinar H, Serre D et al (2004) Genetic analyses from ancient DNA. *Annu Rev Genet* 38:645–679

Cereal Genomics

Methods and Protocols

Henry, R.; Furtado, A. (Eds.)

2014, X, 300 p. 39 illus., 29 illus. in color., Hardcover

ISBN: 978-1-62703-714-3

A product of Humana Press