Genes, Genomes and Genomics ©2008 Global Science Books

In-house Protocol for Isolation of Restrictable and Amplifiable Genomic DNA from Plants, Fungi and Bacteria

Ali H. Bahkali[1] • Kamel A. Abd-Elsalam[1*] • Mohammed A. Moslem[1] • Abdulaziz A. Al-Khedhairy[2]

[1] King Saud University, Faculty of Science, Botany and Microbiology Department, P. O. Box 2455, Riyadh 1145, Saudi Arabia
[2] King Saud University, Faculty of Science, Zoology Department, P.O. Box: 2455, Riyadh 1145, Saudi Arabia

Corresponding author: * **abdelsalamka@gmail.com**

ABSTRACT

Several rapid DNA isolation protocols are not available for plant and microorganisms with the same method. The method was applied to 5 plant species (tomato, cowpea, cotton, date palm and wild mint), 4 fungal species (*Penicillium*, *Trichoderma*, *Fusarium* and *Rhizoctonia*) and 4 bacterial species (*Erwinia*, *Pseudomunas*, *Bacillius* and *Xanthomonas*). Optimal extraction was achieved by incorporating an RNAse A and proteinase K enzymatic digestion step. The protocol produced an acceptable quantity of high-quality DNA. Up to 50 µg of DNA were routinely obtained from a minimal amount of 100 mg of fungal bacterial and plant sample. Fungal DNA prepared by this method was used as a template for PCR to amplify the internal transcribed spacer (ITS) and microsatellite-primed PCR and gave reproducible patterns. The amplicon length of the fragment ITS1/ITS4, ranged in size from 620 to 700 bp. The amplified PCR products of ITS regions in plants ranged in size from 550 to 700 bp, indicating polymorphisms of size in this region. The resultant amplicon was then incubated with the restriction endonucleases *Rsa*I and *Cof*I prior to analysis by gel electrophoresis. The protocol presented here offers an interesting and efficient alternative, eliminating most expensive kits, discounting the material cost per sample to $0.10.

Keywords: 16S ribosomal RNA, biological materials, genomic DNA extraction, pathogenic bacteria, phytofungi
Abbreviations: ITS, internal transcribed spacer; **LB**, Luria-Bertani broth; **PDB**, potato dextrose broth; **PCR**, polymerase chain reaction; **RFLP**, restriction fragment length polymorphism; **SDS**, sodium dodcyle sulphate; **TAE**, tris acetate EDTA; **TE**, tris EDTA

INTRODUCTION

Methods based on DNA analysis can reveal microbial diversity in ecosystems and offer the potential benefits of highly sensitive and rapid detection (Saad *et al.* 2001).

Most commercially available DNA extraction kits from plant, fungi, and bacteria are high costly. The lack of an easy DNA extraction method suited to conditions commonly encountered in many laboratories in developing countries has limited the implementation of DNA analytical services for developing countries (Mahuku 2004).

In setting up analytical PCR, the quality of template DNA is the crucial factor influencing amplification efficiency. A multitude of substances such as proteins, enzymes, chelators, polysaccharides, polyphenols, heparin or urea may be coextracted from contaminated plant material, and fungal and bacterial isolates often inhibited restriction endonuclease digestion and/or PCR amplification (Knoll *et al.* 2002). So far there is no one common and simple procedure for genomic DNA extraction that can be used on a large scale for different eukaryotic organisms.

All methods have in common the use of detergents such as SDS for cell wall lysis, and this often inhibits further purification manipulations. As an alternative to lysis by SDS, toxic chemicals, e.g., phenol, have been used (Zhang and Stewart 2000; Borman *et al.* 2006; Cheng and Jiang 2006).

Up to now, only two research articles reported a DNA extraction method suitable for the efficient extraction of DNA from fungal and plant species (Mahuku 2004; Abd-Elsalam *et al.* 2007). In general different tissues required diverse methods and different tissue preparation steps. Requirement for a universal procedure is urgent, especially when hundreds of samples need to be assayed. The main objective of the present protocol is to provide a simple method of isolation of DNA from plants and microorganisms, using in-house prepared reagents. Expensive and hazardous chemicals were replaced with cheaper, less corrosive materials. This method was developed by combining and modifying the protocols reported by Cenis (1992), De Boer *et al.* (1995), and Möller *et al.* (1992). Also, the procedure involves inactivating proteins by SDS/proteinase K and precipitating polysaccharides in the presence of high salt.

MATERIALS AND METHODS

Plant materials

Young leaves of tomato (*Lycopersicon esculentum*) cv. 'Strain B', cowpea (*Phaseolus vulgaris*) cv. 'GA-21', cotton (*Gossypium barbadense*) cv.'Giza 83', date palm (*Phoenix dactylifera*) cv. 'Bareem' and wild mint (*Mentha arvensis*) cv. 'Bary' were collected from the greenhouse and brought to the laboratory in ice bags. The DNA was isolated from 100 mg of collected fresh leaves on the same day.

Fungal and bacterial isolates

The phytopathogenic bacteria *Erwinia* (PPRI-E3), *Pseudomunas* (PPRI-P19), *Bacillius* (PPRI-B22) and *Xanthomonas* (PPRI-X30), also phytofungi *Penicillium* (PPRI-Pen1), *Trichoderma* (PPRI-Tric5), *Fusarium* (PPRI-Fus11) and *Rhizoctonia* (PPRI-Rhiz15) originally isolated from plant hosts were used in the present analysis. Fungal and bacterial Isolates used in the following studies were obtained from the fungal collection of Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt.

Production of fungal mycelium

To obtain mycelium for DNA isolation from fungal strains, the fungi were grown on potato dextrose broth (PDB) for 7 days at

Received: 24 September, 2008. Accepted: 2 October, 2008.

Original Research Paper

room temperature in darkness without shaking. Mycelia were harvested from a submerged culture by filtration.

DNA extraction from plants and fungi

100 mg from plant leaves and fungal mycelium were ground into fine powder in liquid N_2. Pre-warmed (at 65°C) 500 µL of DNA extraction [SDS method (Cenis (1992): 100 mM Tris–HCl pH 8.0, 50 mM Na_2EDTA pH 8.0, 500 mM NaCl, 1.5% SDS, 0.38% sodium bisulfite] was added to ground samples; also 5 µL of Proteinase K (10 mg/ml) was added and mixed well and incubated for 30 min at 37°C with intermittent mixing every 3 min. The microtube was centrifuged at 13,000 rpm for 15 min and the supernatant was transferred carefully into a new 1.5 ml Eppendorf tube.

130 µl of sodium acetate solution (3 M, pH 5.52) was added and incubated at -20°C for 20 min. The Eppendorf tube was centrifuged at 13,000 rpm for 15 min. The upper aqueous phase was decanted into fresh centrifuge tubes and DNA was precipitated with 2/3 volume of ice-cold isopropanol and kept at room temperature for 7 min. DNA was pelleted down by centrifugation at 4°C for 10 min at 10,000 rpm. The DNA pellet was washed two times with 70% ethanol (700 µl) and centrifuged at 8000 rpm for 1 min, vacuum dried and dissolved in 100 µl of warmed TE buffer. Finally, 5 µl RNAse A (20 mg/ml) was added and incubated at 37°C for 30 min.

DNA extraction from bacteria

To extract genomic DNA from pure culture, bacterial cells were grown in Luria-Bertani broth (LB) at 28°C for 24 to 48 h until confluent growth was obtained. 250 µl of such 48-hr old cultures were then harvested by centrifugation (8000 rpm, 5 min) and the pellet obtained was washed twice with distilled water, and recentrifuged. 50 µl of PCR buffer (New England Biolab, UK) and 1 µl Proteinase K (10 mg/ml) was added and the whole mixture was vortexed to ensure good homogenization, and then incubated the microtube at -20°C overnight. The frozen samples were defrosted for 60 min in a warm heated water bath (60°C) and the suspension was incubated for 10 min at 95°C. To finish, 5 µl RNAse A (10 mg/ml) was added and incubated at 37°C for 20 min. All extracted DNA was stored at -20°C until use.

Quality of the extracted DNA

4 µl of the isolated DNA and 3 µl of sterile distilled water were mixed with 3.0 µl of 10 X loading dye and was loaded in a lane of 1% (w/v) agarose gel for verifying the quality of the DNA. The intact dsDNA forming a thick single band of high molecular weight confirmed the high-quality of the extracted DNA.

Amplification and digestion of ITS rDNA

After the extraction of gDNA from fungal and plant species, the ITS was amplified using the ITS1/ITS4 and NS3/NS4 primers designed for the amplification of specific regions of the rDNA respectively. The amplification reactions were performed in 25-µl reaction volumes containing 100 ng of template DNA, 25 mM $MgCl_2$, 2 mM dNTPs (JenaBioscience, Germany), 10 pmol of each primer (MWG, Germany), and 1 U Taq polymerase (JenaBioscience, Germany) using previously published PCR parameters (Abd-Elsalam *et al.* 2007). Primers sequence used the present study are listed in **Table 1**.

Digestion of PCR product and genomic DNA

PCR amplicons (NS3/NS4) from plants were mixed with 2.5 µl of enzyme buffer, 0.5 µl *Rsa*I and 0.5 µl *Cof*I restriction enzymes (Roche, Germany) and digested at 37 C for 60 min. 4 µg of bacterial genomic DNA was incubated overnight with mixture of 5 U *Rsa*I and 5 U *Cof*I. The resulting fragments were visualized after electrophoresis in a 1.5% agarose gel, run in TAE buffer as above.

Microsatellite analysis

PCR products were obtained in a total volume of 25 ml with 20 ng of template DNA, 0.2 mM of T3B primer (MWG, Germany), 200 mM of each dNTP, 1 U Taq Polymerase (JenaBioscience, Germany) and 1X reaction buffer for used polymerase. DNA and PCR mixture were amplified in Techne TC-312 (Techne, Stone, UK) under the following conditions: initial denaturation at 94°C for 3 min; 40 cycles: denaturation at 94°C for 20 s, annealing at 50°C for 1 min, extension at 72°C for 20 s and a final extension at 72°C for 7 min. Amplification products were resolved electrophoretically on 1.5% agarose gel in 1X TAE buffer by loading 8 µl into prepared wells. Gels were stained with ethidium bromide.

Gel documentation

All agarose gels were stained with ethidium bromide (1 µg/ml in distilled water) for 15 min at room temperature and inspected in transmittant UV light at 233 nm, and photographed by Gel Documentation System (Uvitec, Cambridge, UK). ImageForge software was employed for processing digital images of the gels.

Costs calculation

The cost per sample for each DNA extraction protocol was determined. The list prices of chemicals and consumables supplied by the manufacturers were use, which results in the maximum price per extraction.

RESULTS

Only one major band was visualized on the agarose gel, and all seemed longer than 3000 bp, no smearing DNA appeared on the gel though the marker fragment of 100 bp was clearly visible (**Fig. 1**). In order to check the efficiency and reliability of the method, we first amplified the genomic DNA of fungal species using microsatellite primer T3B. The amplified PCR products of fungal genomic DNA showed distinguishable band patterns (**Fig. 2**). These microsatellite analysis results proved that the extracted DNA is useful for PCR amplification. On amplification using ITS primers the isolated DNA showed high intensity bands. The PCR products obtained from the rDNA amplification were of approximately 620-700 bp in different fungal species (**Fig. 3**). In plants, the 550 bp to 700 bp PCR fragments amplified using primers N3and NS4 were digested using the restriction enzymes *Rsa*I and *Cof*I. The tested enzymes *Rsa*I, and *Cof*I produced polymorphisms among the plant species assayed. The PCR-RFLP profiles obtained with tested enzymes differentiated plant species (**Fig. 4**).

The DNA extracts from bacteria were then digested with *Rsa*I and *Cof*I. Perfect smears were observed (**Fig. 5**). RNase treatment was sufficient for degrading RNA into small ribonucleosides that do not contaminate the DNA preparation, as was evident in the agarose gel electrophoresis (**Fig. 6**).

The protocol is cost-effective, with a reagent cost per sample as low as $0.10 although the costs of labor, reagents, and plastic consumables vary dramatically over time and between countries.

Table 1 Primers list used in the current study

Primer name	Sequence 5′-3′	target DNA	Reference
ITS1	TCCGTAGGTGAACCTGCGG	Fungal species	White *et al*. 1990
ITS4	TCCTCCGCTTATTGATATGC		
NS3	GCAAGTCTGGTGCCAGCAGCC	Plant species	Kuske *et al*. 1998
NS4	CTTCCGTCAATTCCTTTAAG		
T3B	AGGTCGCGGGTTCGAATCC	Fungal species	Alves *et al*. 2007

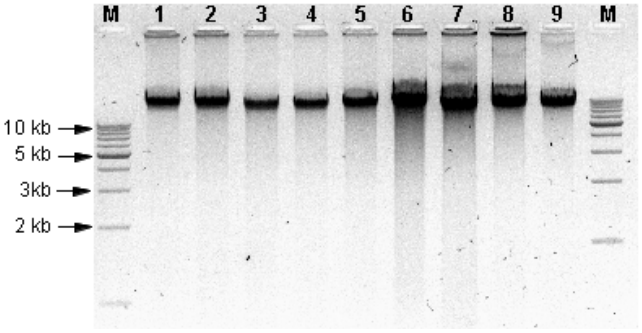

Fig. 1 Genomic DNA profile extracted from different plants and fungi. Lane 1, tomato cv. 'Strain B'; Lane 2, cowpea cv. 'GA-21'; Lane 3, date palm cv. 'Bareem'; Lane 4 cotton cv.'Giza 83'; Lane 5, wild mint cv. 'Bary'; Lane 6, *Penicillium* (PPRI-Pen1); Lane 7, *Trichoderma* (PPRI-Tric5); Lane 8, *Fusarium* (PPRI-Fus11); Lane 9, *Rhizoctonia* (PPRI-Rhiz15). Lane M, 1-kp DNA ladder size marker (Jena Bioscience).

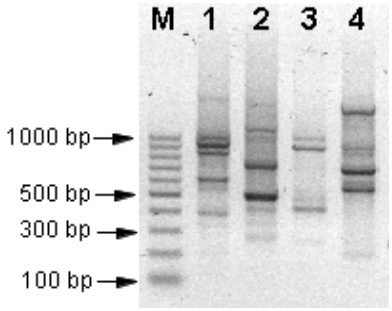

Fig. 2 Microsatellite-primed PCR (MP-PCR) amplification from various fungal species using DNA extracted following the present method. Lane 1, *Penicillium* (PPRI-Pen1); Lane 2, *Trichoderma* (PPRI-Tric5); Lane 3, *Fusarium* (PPRI-Fus11); Lane 4, *Rhizoctonia* (PPRI-Rhiz15). Lane M, 100-bp DNA ladder size marker (Jena Bioscience).

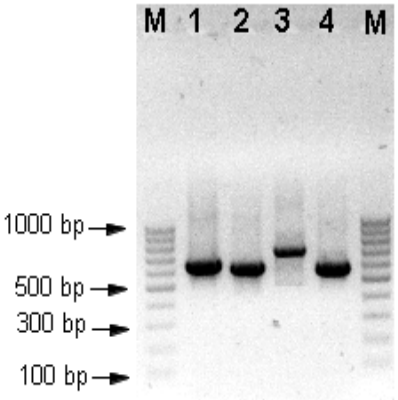

Fig. 3 PCR amplification of the ITS region from various fungal species using DNA extracted following the present method. Lane 1 *Penicillium* (PPRI-Pen1); Lane 2, *Trichoderma* (PPRI-Tric5); Lane 3, *Fusarium* (PPRI-Fus11); Lane 4, *Rhizoctonia* (PPRI-Rhiz15). Lane M, 100-bp DNA ladder size marker (Jena Bioscience).

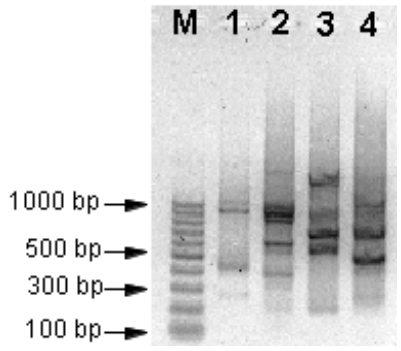

Fig. 4 *Rsa*I + *Cof*I digest of the PCR-amplified portion of rDNA using the primers NS3 and NS4 from different plants. Lane 1, tomato cv. 'Strain B'; Lane 2, cowpea cv. 'GA-21'; Lane 3, date palm cv. 'Bareem'; Lane 4 cotton cv.'Giza 83'. Lane M, 100-bp DNA ladder size marker (Jena Bioscience).

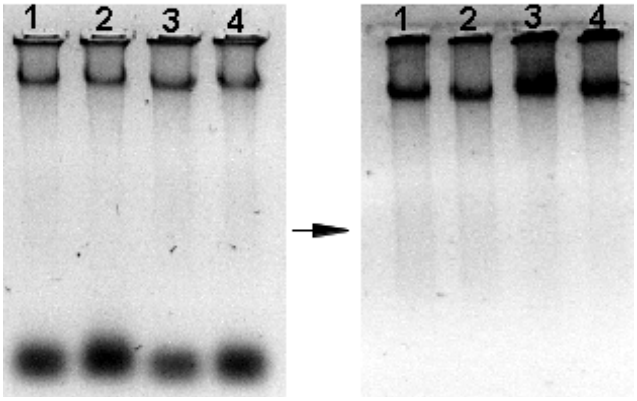

Fig. 5 Bacterial DNA non-treated (left gel) and treated (right gel) with RNase A. Lane 1, *Erwinia* (PPRI-E3); Lane 2, *Pseudomounas* (PPRI-P19); Lane 3, *Bacillius* (PPRI-B22); Lane 4, *Xanthomonas* (PPRI-X30).

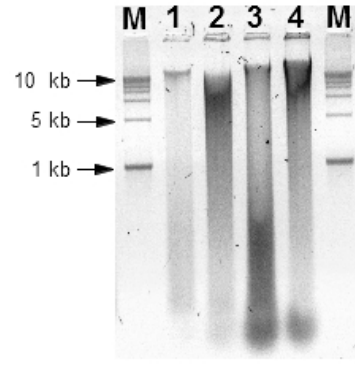

Fig. 6 Bacterial DNA digested with mixture of two restriction endonucleases *Rsa*I + *Cof*I. Lane 1, *Erwinia* (PPRI-E3); Lane 2, *Pseudomonas* (PPRI-P19); Lane 3, *Bacillius* (PPRI-B22); Lane 4, *Xanthomonas* (PPRI-X30).Lane M, 1-kp DNA ladder size marker (Jena Bioscience).

DISCUSSION

The selection of a DNA extraction method is dependent on several factors, including assay targets (RNA/DNA-based), specimen type, sample throughput, laboratory workflow, costs, and, foremost, and maximum number of samples extracted must be considered.

The general aim was to develop an isolation procedure that could be performed using materials common to most science laboratories and would provide DNA of sufficient quality to be used in subsequent molecular biology applications. The steps involved were (1) grinding of samples, (2) DNA extraction and precipitation, and (3) removing RNA contamination by RNase. We combined proteic (i.e., proteinase K) and mechanical (i.e., liquid nitrogen) lysis to obtain an acceptable quantity and quality of fungal DNA. Previous experience had indicated that grinding in liquid nitrogen was entirely sufficient to disrupt both plant and fungal tissues (Nazar *et al.* 1991). The protocol was standardized for 100-mg samples, which can be handled in a 1.5-mL disposable Eppendorf tube. The DNA extracted from plant, fungal and bacterial samples consisted largely of long fragments, i.e. longer than 3000 bp, indicating that a large part of the DNA did not break into small fragments during the extraction procedures. The DNA resulting from the optimized protocol was observed to be largely free from polyphenolics and secondary metabolites, protein and RNA. Most proteins are removed by means of treatment of the

extract with heat-treated RNase A. RNase treatment is the generally accepted method to remove RNA; however, degradation is often incomplete. The contaminated RNA that precipitates along with DNA causes many problems including suppression of PCR amplification (Pikbart and Villeponteau 1993). Addition of 3 M sodium acetate and ethanol increased the solubility of polysaccharides thereby effectively decreasing co-precipitation of the polysaccharides and DNA. All methods have in common the use of detergents such as SDS for cell wall lysis, and this often inhibits further purification manipulations (Melo *et al.* 2006). PCR amplification of the isolated genomic DNA was successful with two molecular markers techniques. The restriction profile generated from the ITS region showed high degrees of polymorphisms for all the plant tested. The species studied had different digestion profiles with the tested enzymes as well as different amplicon sizes. The material costs for our extraction method were five- to six-fold cheaper than the materials for the previous method, reducing the cost per sample to $0.10. Teixeira da Silva (2005) found, in plant (tobacco, chrysanthemum and *Spathiphyllum*) analyses comparing 12 extraction methods and commercial kits that costs of DNA extraction protocols could differ as much as 61-fold, depending on the extraction method, with the lowest method costing 0.08$ per sample. Furthermore, use of this protocol will allow researchers to obtained DNA from plants, fungi and bacteria quickly for molecular assays and replaces expensive and consuming procedures. The proposed protocol makes use of in-house prepared and readily available reagents and thus provides an alternative to the use of commercial DNA isolation kits.

ACKNOWLEDGEMENTS

This work was financially supported by research grant from King Saud University, College of Science, Research Center Project No (Bot/2008/12). The analyses were carried out at the Molecular Mycology Laboratory of the Botany and Microbiology Department, College of Science, KSU.

REFERENCES

Abd-Elsalam KA, Asran-Amal A, El-Samawaty AMA (2007) Isolation of high quality DNA from cotton and its fungal pathogens. *Journal of Plant Disease and Protection* **114**, 113-116

Alves A, Phillips AJL, Henriques I, Correia A (2007) Rapid differentiation of species of Botryosphaeriaceae by PCR fingerprinting. *Research Microbiology* **158**, 112-121

Borman AM, Linton CJ, Miles SJ, Campbell CK (2006) Ultra-rapid preparation of total genomic DNA from isolates of yeast and mould using Whatman FTA filter paper technology - a reusable DNA archiving system. *Medical Mycol*ogy **44**, 389-398

Cenis JL (1992) Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Research* **20**, 238

Cheng HR, Jiang N (2006) Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnology Letters* **28**, 55-59

De Boer SH, Ward, LJ, Chittaranjan S (1995) Attenuation of PCR inhibition in the presence of plant compounds by addition of BLOTTO. *Nucleic Acids Research* **23**, 2567

Knoll S, Mulfinger S, Niessen L, Vogel RF (2002) Rapid preparation of *Fusarium* DNA from cereals for diagnostic PCR using sonification and an extraction kit. *Plant Pathology* **51**, 728-734

Kuske CR, Banton KL, Stark PC, Hill KK, Jackson J (1998) Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. *Applied and Environmental Microbiol*ogy **64**, 2463-2472

Mahuku GS (2004) A simple extraction method suitable for PCR based analysis of plant, fungal, and bacterial DNA. *Plant Molecular Biology Report* **22**, 71-81

Melo SCO, Pungartnik C, Cascardo JCM, Brendel M (2006) Rapid and efficient protocol for DNA extraction and molecular identification of the basidiomycete *Crinipellis perniciosa. Genetics and Molecular Research* **5**, 851-855

Möller EM, Bahnweg G, Sandermann H, Geiger HH (1992) A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Research* **20**, 6115-6116

Nazar RN, Hu X, Schmidt J, Culham D, Robb EJ (1991) Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of verticillium wilt pathogens. *Physiological and Molecular Plant Pathology* **39**, 1-11

Pikbart MJ, Villeponteau B (1993) Suppression of PCR amplification by high levels of RNA. *Biotechniques* **14**, 24-25

Saad DS, Kinsey GC, Kim S, Gaylarde CC (2001) Molecular methods for the analysis of fungal growth on painted surfaces. In: *Proceedings of the 4th Latin American Biodeterioration and Biodegradation Symposium*, 16–20 April, 2001, Buenos Aires, published on CD-ROM

Teixeira da Silva JA (2005) Effectiveness of DNA extraction protocols for horticultural and physiological model plant analyses. *International Journal of Botany* **1**, 93-99

White TJ, Burns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfald DH, Sninsky JJ, White TJ (Eds) *PCR Protocol: A Guide to Methods and Applications*, Academic Press, New York, USA, pp 315-322

Zhang J, Stewart J (2000) Economical and rapid method for extracting cotton genomic DNA. *Journal of Cotton Science* **4**, 193-201