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TECHNICAL NOTES

Preservation of DNA in plant specimens: inactivation and re-activation of DNases in field specimens

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The preservation of plant specimens by silica gel desiccation for subsequent DNA analysis is now routine (Adams *et al*. 1992). The Missouri Botanical Garden Herbarium even has a DNA bank that consists of silica gel-dried materials (Miller & Schmidt 1998). It is thought that DNases (proteins) are denatured by the extreme desiccation produced by dry silica gel (Adams *et al*. 1992). The most common DNA extraction protocol used in plant science appears to be the hot CTAB method (Doyle & Doyle 1987). In this and most other protocols, EDTA is a component because of its ability to chelate Mg^{2+} ions, which are often needed by DNases (Ogawa & Kuroiwa 1985). However, the assumption that all DNases can be inhibited by EDTA is not warranted. For example, in *Clamydomonas reinhardtii*, six DNases were found and each of these required Ca^{2+} for activation (Ogawa & Kuroiwa 1985). The DNases were little effected by the amount of Mg^{2+} ions. In tobacco, the DNases did not appear to need any specific ions for activity and were inhibited by Mg^{2+} (Zilberstein *et al*. 1987). Two DNases were found in wheat seedlings (Jones & Boffey 1984): one required Mg^{2+} and the other was activated by EDTA. Jones & Boffey (1984) concluded: 'Thus, EDTA alone will not protect DNA from cleavage during its isolation from wheat seedlings.'

The DNases are even more complex in rice. Sodmergen *et al*. (1991) found that rice contained 13 DNases with the following ion requirements: five Ca^{2+}, four Zn^{2+}, and four Mg^{2+} dependent. If EDTA only chelates Mg^{2+} ions, it would not be effective in inhibiting these DNases.

We recently received some vetiver (*Vetiveria*, Gramineae) samples from Madagascar that were shipped in silica gel in resealable plastic bags. The blue indicating crystals had turned partially pink (implying that some re-hydration had occurred). The DNAs from these samples were very degraded. It appears that the DNases in vetiver may be re-activated by the addition of water. In fact, we have encountered difficulty in obtaining good DNA from vetiver by grinding directly in CTAB (Adams *et al*. 1998). It has been necessary to grind in liquid nitrogen and then regrind in CTAB. We thought this problem was due to the fibrous nature of the *Vetiveria* leaves (particularly when dry) which caused it to be difficult to grind to a fine powder in CTAB. However, we experienced difficulty in obtaining DNA of uniform quality, even when extracting fresh vetiver leaves which are easy to grind. It seemed plausible that the DNases were re-activated by water in the extraction buffer and that the EDTA was ineffective against these DNases.

Because different plant species apparently produce different kinds of DNases, it seems that a more general method for DNase inactivation is needed. Previously, we reported on the effectiveness of various alcohols in preserving plant specimens (Flournoy *et al*. 1996). The alcohols apparently precipitated the proteins (including DNases) and, thus, protected the DNA. Ethanol was found to be the most effective alcohol tested (Flournoy *et al*. 1996).

The purpose of this study was to investigate the effects of ethanol on DNases from both fresh and silica gel-dried materials from several plant families to find a more general method for the extraction of preserved materials.

Leaves from fresh spinach (*Spinacia oleracea* L.), broccoli (*Brassica oleracea* L.), and alfalfa sprouts (*Medicago sativa* L.) were purchased locally. Juniper (*Juniperus virginiana* L.) leaves were collected from trees cultivated near the laboratory. Sorghum (*Sorghum bicolor* (L.) Moench.), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), and rice (*Oryza sativa* L.) seed were secured locally and germinated to obtain seedlings.

Plant material was ground in a micromortar in 100 µL of either double-distilled water (dd water), CTAB, or ethanol. The sample was then incubated for 20 min at 37 °C as a DNase activity assay (in the case of ethanol, 350 µL of dd water was added before incubation). Then, 350 µL of hot CTAB (Doyle & Doyle 1987) was added to the CTAB- or ethanol-extracted samples. Proteinase (150 µg, Sigma P6911) was added and all the samples were incubated for an additional 30 min at 60 °C. The DNA was precipitated by the addition of 2 vols of ethanol (rather than the use of 2/3 vols of isopropanol in Doyle & Doyle (1987). DNA was separated on 0.6% agarose gels by electrophoresis (20 min, 100 V, 10 V/cm) with ethidium bromide in the gel and buffer. DNA quantities and qualities were estimated by comparisons with serial dilutions of genomic mouse DNA (Sigma D-0144) and lambda *Hin*dIII markers. Gels were photographed in short-wave UV light using a Polaroid direct screen camera (DS34). In addition, all the extracted DNAs were subjected to PCR–random amplified polymorphic DNA (RAPDs) (Adams *et al*. 1998) using a standard RAPD primer (UBC 268) to check for the quality of the DNAs.

All of the species contained DNases that degraded the DNA when the ground material was incubated in dd water for 20 min at 37 °C (Table 1), except juniper, in which case the DNA was only partially degraded but completely degraded after 24 h at 37 °C. Note particularly that preservation in silica gel does not irreversibly inactivate DNases. In every case, except broccoli, the DNA in silica gel-dried leaves was degraded when the leaves were ground in water and incubated (Table 1). Thus, it appears that when shipping materi-

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als, one must be very careful that the materials are not rehydrated either during transit or prior to extraction.

All of the nongrasses yielded very good DNA when ground in CTAB and then incubated in CTAB (20 min, 37 °C). In contrast, CTAB was either not very effective or ineffective in protecting the DNA for most of the grasses (Table 1, Fig. 1). Only the fresh maize and fresh wheat yielded very good DNA (20–50 kbp) when ground in CTAB and incubated. Fresh sorghum and silica-dried wheat yielded good (some degraded DNA) under these conditions.

However, even the most recalcitrant species (vetiver and rice) yielded very good DNA (Table 1) when the materials were first ground in ethanol and then incubated in dd water (20 min, 37 °C). It seems that the ethanol, in precipitating the proteins, has also irreversibly denatured the DNases.

All of the samples that yielded good or very good DNAs (Table 1) produced good bands with PCR–RAPD, whereas those with poor or degraded DNAs either failed to amplify or produced variable bands.

Table 1 Comparison of the DNA obtained from leaves ground in CTAB, dd water or ethanol. The dd water and ethanol grindings were further incubated in dd water for 20 min, 37 °C, before adding CTAB and incubating for 30 min, 60 °C

	Quality of DNA from leaves ground in		
Material	dd water	CTAB	Ethanol
Fabaceae			
alfalfa, fresh	– –	+ +	+ +
alfalfa, silica dried	– –	+ +	+ +
Cruciferae			
broccoli, fresh	– –	+ +	+ +
broccoli, silica dried	+	+ +	+ +
Cupressaceae			
juniper, fresh	+ (– ,24 h)	+ +	+ +
juniper, silica dried	– –	+ +	+ +
Chenopodiaceae			
spinach, fresh	– –	+ +	+ +
spinach, silica dried	– –	+	+ +
Gramineae (grasses)			
maize, fresh	– –	+ +	+ +
maize, silica dried	–	–	+ +
sorghum, fresh	– –	+	+ +
sorghum, silica dried	– –	–	+ +
rice, fresh	– –	– –	+ +
rice, silica dried	– –	–	+ +
vetiver, fresh	– –	– –	+ +
vetiver, silica dried	– –	– –	+ +
wheat, fresh	– –	+ +	+ +
wheat, silica dried	– –	+	+ +

DNA quality: + + = very good, molecular weight (MW) of 20–50 kbp; + = good, MW of 20–50 kpb, but some degraded DNA on gel ranging down to 200–300 bp; – = poor, essentially no DNA of MW 20–50 kbp, DNA smeared from 6kbp to 200–300 bp; – – = degraded DNA, MW of only 200–300 bp.

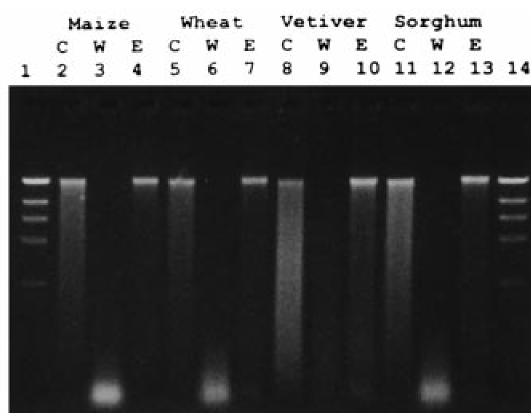

Fig. 1 Effects of grinding buffer on DNA quality. C = CTAB used in grinding, W = dd water used in grinding, E = ethanol used in grinding. All materials were dried in silica gel, 72 h, 22 °C before extraction. Lanes 1 and 14, lambda/*Hin*dIII markers; lanes 2–4, maize; lanes 5–7, wheat; lanes 8–10, vetiver; lanes 11–13, sorghum.

The grinding of plant material in a small quantity of ethanol, before grinding in the extraction buffer (CTAB in this instance), would seem to be a general method for the inactivation of DNases, regardless of their requirements for Mg^{2+}, other ions or no ions.

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References

Adams RP, Do N, Chu G-L (1992) Preservation of DNA in plant specimens from tropical species by desiccation. In: *Conservation of Plant Genes: DNA Banking and in Vitro Biotechnology.* (eds Adams RP, Adams JE), pp. 135–152. Academic Press, San Diego.

Adams RP, Zhong M, Turuspekov M, Dafforn MR, Veldcamp JF (1998) DNA fingerprinting reveals clonal nature of *Vetiveria zizanioides* (L.) Nash, Gramineae and sources of potential new germplasm. *Molecular Ecology*, **7**, 813–818.

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, **19**, 11–15.

Flournoy LE, Adams RP, Pandey N (1996) Interim and archival preservation of plant specimens in alcohol for DNA studies. *Biotechniques*, **20**, 657–660.

Jones MC, Boffey SA (1984) Deoxyribonuclease activities of wheat seedlings. *Federation of European Biochemical Societies*, **174**, 215–218.

Miller J, Schmidt H (1998) The Missouri Botanical Garden's DNA Bank. In: *Conservation of Plant Genes III: Conservation and Utilization of African Plants.* (eds Adams RP, Adams JE), pp. 175–182. Missouri Botanical Garden Press, St Louis, MO, USA.

Ogawa K, Kuroiwa T (1985) Nuclease C: polymorphism of calcium-dependent nucleases in *Clamydomonas reinhardtii*. *Plant Cell Physiollogy*, **26**, 481–491.

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Sodmergen K, Kawano S, Tano S, Kuroiwa T (1991) Degradation of chloroplast DNA in second leaves of rice (*Oryza sativa*) before leaf yellowing. *Protoplasma*, **160**, 89–98.

Zilberstein A, Koch T, Altschuler Y, Lers A, Zamir A (1987) Characterization of DNase (s) activity in tobacco leaf extracts. *Plant Science*, **52**, 57–65.

An ultrasensitive method for detection of single crab larvae (*Sesarma reticulatum*) by PCR amplification of a highly repetitive DNA sequence

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For studies of planktonic organisms, even the basic question of whether a particular species is represented in a plankton sample may be difficult to answer. Traditional methods of morphological identification by microscopy are labour intensive and require species-specific diagnostic characters (Silberman & Walsh 1992; Fuhrman *et al*. 1994; Medeiros-Bergen *et al*. 1995). These characters may be absent, highly variable, or obscured by specimen damage during collection (Smith 1977; Medeiros-Bergen *et al*. 1995). Studies of planktonic communities would thus benefit from methods of identification that do not depend on morphology. Here we report a novel approach for the detection of larval *Sesarma reticulatum* (marsh crab) that is based on the presence of a characteristic repetitive sequence element. This sequence element is ≈ 600 bp in length, represented by about 5×10^5 copies per haploid genome, and bears no significant similarity to sequences in the GenBank database (release 108). Because of its high copy number, it can be detected in samples that contain less DNA from *S. reticulatum* than is present in a single cell (Rheinsmith *et al*. 1974).

A small genomic library for *S. reticulatum* was created in the plasmid vector pUC18. A large percentage of the sequences in this library exhibited similar restriction site maps and cross-hybridized on Southern blots (methods described in Neigel *et al*. 1991). Partial sequence analysis of several of these plasmids confirmed that they contained overlapping portions of a repetitive sequence, which we designated Sr16.

Using the computer program NAR (Rychlik & Rhoads 1989), we designed a pair of PCR primers to amplify a 294-bp region of Sr16, hereafter designated as Sr16-PCR. Primers were synthesized by BioSynthesis, Inc. (Lewisville, Texas) and are: SR16F-F1, 5'-ATCGAAGAATAAGAGGCGACT-3'; SR16F-R1, 5'-ACGTAATTAACCGAGCATTGA-3'.

Amplification conditions were optimized for these primers, and used for all subsequent experiments. The optimized amplification profile was 35 cycles of 1 min at 94 °C, 1 min at 50 °C, ramp to 72 °C for 2 min, and 1.5 min at 72 °C. Each 25 µL reaction included 10 pM of each primer and final concentrations of 2 mM of each dNTP; 2.5 mM $MgCl_2$; 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin; 1.25 U *Taq* polymerase (Boehringer Mannheim); and template DNA (varying concentrations). Reaction mixtures were covered with a layer of sterile mineral oil to reduce evaporation.

Genomic DNA was extracted from muscle tissue of adult crabs by phenol/chloroform extractions and from larval crustaceans with 'protocol A for larval extraction' and 'protocol B – alcoholic tissues' (Palumbi *et al*. 1991). For extractions of individual larval *S. reticulatum*, 25 µL of naupliar larval *Artemia* sp. (Ocean Star International, Inc.) was added as a 'carrier' to provide a visible quantity of material. Each PCR experiment consisted of three replicates and a negative control that lacked template DNA. Amplification products were electrophoresed through 2% agarose/Tris-borate EDTA gels alongside a DNA size standard and then visualized by staining with 25 µg/mL ethidium bromide and UV transillumination (Maniatis *et al*. 1982). A reaction was scored as positive if a product of the same mobility as Sr16-PCR was observed.

PCR theoretically can detect a single DNA molecule; in combination with this extreme sensitivity the high copy number of Sr16 provided the basis for an ultrasensitive method for detection of genomic DNA from *S. reticulatum* (SrDNA). Sr16-PCR was consistently amplified from quantities of SrDNA that spanned five orders of magnitude (Table 1), including quantities below the haploid genome size of 4.1 pg measured for the closely related species *Armases cinereum* (see Rheinsmith *et al*. 1974).

Amplifications of Sr16-PCR were also successful when SrDNA was mixed with DNA from other species. In mixtures with DNA from larval *Artemia* sp., it was possible to amplify Sr16-PCR from as little as 1 pg of SrDNA, although the concentration of DNA from *Artemia* was 10 000-fold higher (Table 1). This sensitivity provided consistent detection of DNA from single larval *S. reticulatum* that were coextracted with 25 µL of *Artemia* (about 500 nauplii). Amplifications were also successful in the presence of DNA extracted from a

Table 1 Detection of genomic DNA from *Sesarma reticulatum* mixed with DNA from *Artemia*. Numbers in parentheses are the mass ratios of DNA from *S. reticulatum* to DNA from *Artemia*

	Artemia	
Sesarma	1 ng	10 ng
10 ng	+ (10:1)	+ (1:1)
1 ng	+ (1:1)	+ (1:10)
0.01 ng	NA	+ (1:1000)
0.001 ng	NA	+ (1:10 000)

+ indicates successful amplification in all three replicates; NA indicates amplification was not attempted.

brachyuran crab (*Menippe*) and a sample of freshwater plankton (Cypress Lake, LA). Sr16-PCR was not amplified (at detectable levels) in controls with DNA from these other species alone.

For accurate identification, specificity of the PCR amplification as well as sensitivity is essential. Species with sequences similar to Sr16 could generate 'false positives' if PCR products similar in size to Sr16-PCR were amplified. However, although many other species could potentially occur with *S. reticulatum* in plankton samples, similar sequences would most probably occur in related species. We therefore tested six other species from the family Grapsidae, including species closely related to *S. reticulatum*. None generated false positives (Table 2).

Sensitive detection of SrDNA was not dependent on the method of sample preservation. Amplifications were successful with larvae frozen immediately after collection or preserved in either glycerol or 95% ethanol. Prior to extraction, glycerol was removed by four rinses with TE (20 mM Tris, pH 7.5, 100 mM EDTA).

Repetitive sequences such as Sr16 are seldom used as taxonomic characters because they are subject to unusual modes of evolution (Arnheim 1983), and homologous sequences are often restricted to closely related species (Felger & Hunt 1993). However, the variability of repetitive sequences could make them ideal species tags. They are easier to detect than low-copy sequences and, if characteristic of a particular species, their presence alone is sufficient to identify that species. Much greater effort and expense is required to characterize nuclear or mitochondrial sequences when used for species identification. We expect that the approach described here can be applied to other species and will be useful for quantification as well as detection of planktonic organisms.

Table 2 Species of grapsid crabs tested for false-positive amplification of a PCR with primers for Sr16-PCR

Species	Product	Relationship
Sesarma reticulatum (+ control)	+	Self
Sesarma cuacaoense	–	Congener
Sesarma fossarum	–	Congener
Armases cinereum	–	Same subfamily
Cyclograpsus integer	–	Same subfamily
Hemigrapsus oregonensis	–	Different subfamily
Pachygrapsus transversus	–	Different subfamily

+ indicates that a product of the expected size was amplified;
– indicates that no discrete product was amplified.

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References

Arnheim N (1983) Concerted evolution of multigene families. In: *Evolution of Genes and Proteins* (eds Nei M, Koehn RK), pp. 38–61. Sinauer Associates, Sunderland, MA.

Felger I, Hunt JA (1993) Detection and characterization of transposable elements. *Methods in Enzymology*, **224**, 322–334.

Fuhrman JA, Lee SH, Masuchi Y, Davis AA, Wilcox RM (1994) Characterization of marine prokaryotic communities via DNA and RNA. *Microbiology and Ecology*, **28**, 133–145.

Maniatis T, Gritsch EF, Sambrook J (1982) *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.

Medeiros-Bergen DE, Olsen RR, Conroy JA, Kocher TD (1995) Distribution of holothurian larvae determined with species-specific genetic probes. *Limnology and Oceanography*, **40**, 1225–1235.

Neigel JE, Felder DL, Chlan CA, La Porte R (1991) Cloning and screening of DNA probes for genetic studies in stone crabs (Decapoda: Xanthidae: *Menippe*). *Journal of Crustacean Biology*, **11**, 496–505.

Palumbi SR, Martin A, Romano S, McMillan WO, Stice L, Grabowski G (1991) *The Simple Fool's Guide to PCR*. University of Hawaii, Honolulu.

Rheinsmith EL, Hinegardner R, Bachman K (1974) Nuclear DNA amounts in Crustacea. *Comparative Biochemical Physiology*, **48B**, 343–348.

Rychlik W, Rhoads RE (1989) A computer program for choosing optimal oligonucleotides for filter hybidization, sequencing and *in vitro* amplification of DNA. *Nucleic Acids Research*, **17**, 8543–8551.

Silberman JD, Walsh PJ (1992) Species identification of spiny lobster phyllosome larvae via ribosomal DNA analysis. *Molecular Marine Biology and Biotechnology*, **1**, 195–205.

Smith DL (1977) *Marine Coastal Plankton and Marine Invertebrate Larvae*. Kendal/Hunt Publishing, Dubuque, Iowa.