Seed Science Research (2006) **16**, 169–178 DOI: 10.1079/SSR2006246

The utility of aged seeds in DNA banks

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Abstract

The long-term utility of DNA banks is predicated on the stability of DNA during storage. The quality and yield of DNA extracted from seeds from four garden species, which varied in age from 1 to 135 years old, was used to examine the early stages of DNA degradation. Seeds that were 70 years old yielded high molecular weight DNA, which permitted amplification of a 650 bp segment of the internal transcribed spacer (*ITS*) region of the nuclear ribosomal DNA and a 487 bp segment of the plastid-encoded large subunit of rubisco (*rbcL*). DNA extracted from seeds estimated to be about 135 years old was degraded, but still permitted amplification when routine extraction procedures were used. The *rbcL* sequences obtained from *c.* 135-year-old cucurbit seed DNA were consistent with species identifications based on seed morphological features; however, *ITS* sequences from the same samples were determined to be of fungal origin. A comparison of our results with studies of DNA stability in leaf specimens suggested that DNA degraded within both tissues, but perhaps more slowly within seeds. Evidence that high-quality DNA can be extracted from old, non-viable seeds expands the utility of seed banks in preserving genetic resources.

Keywords: DNA banks, genetic resources, ITS sequences, ribosomal DNA, rubisco, seed ageing, seed DNA stability

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This work was funded by institutional sources. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

Introduction

DNA banks provide a source of genetic information for research about gene function, evolution, taxonomy, population history and epidemiology. Well-established for human medical applications (e.g. Madisen *et al.*, 1987; Austin *et al.*, 1996), DNA banks are increasingly in demand for plants and animals (e.g. Savolainen and Reeves, 2004). DNA sequences obtained from banked DNA can be used to evaluate patterns of genetic variation within and among species, map and characterize desirable gene complexes, and infer the evolution of genome structure (e.g. Gugerli *et al.*, 2005). In order to provide useful materials for genomic surveys, DNA banks must ensure that high-quality (high molecular weight and purity) DNA can be maintained for many years.

Seed banks can be readily adapted to the needs of DNA banks. Seeds are efficient and inexpensive vessels for storing DNA of individual genotypes. Large quantities of high molecular weight DNA can be extracted from live seeds or from seedling tissues generated during routine viability tests. If seeds are viable, the supply of DNA can be replenished. However, seeds inevitably lose viability during storage (e.g. Walters *et al.*, 2005). Low-viability samples are often decommissioned and discarded. The presence of high-quality DNA within dead tissues may allow extension of the useful lifespan of non-viable seed accessions. This may be of particular importance for accessions collected from wild populations (where high costs may prevent repeat collections), or accessions known to contain individuals with novel genotypes.

To evaluate whether non-viable seeds may be useful for DNA banking, the long-term stability of DNA in seeds must be evaluated. Damage to DNA structure has been reported during ageing or drying of both desiccation-tolerant and intolerant organisms (Cheah and Osborne, 1978; Roberts, 1978, 1988; Murata *et al.*, 1980; Dandoy *et al.*, 1987; Whittle *et al.*, 2001; Osborne *et al.*, 2002; Phillips *et al.*,

2002; Shirkey *et al.*, 2003), suggesting that DNA degradation is linked to death, or that DNA degradation continues under standard seed storage conditions. However, changes of DNA that cause death (i.e. loss of telomere repeats or unrepaired breaks) are minor, compared to the fragmentation that occurs after death or even during simple extraction procedures. Even after death, DNA is extremely stable in seeds, as is demonstrated by the retrieval of genetic information from >1000-year-old specimens found in archaeological sites (Pääbo *et al.*, 1989; Rollo *et al.*, 1991; Goloubinoff *et al.*, 1993; Allaby *et al.*, 1994, 1997; O'Donoghue *et al.*, 1996; Deakin *et al.*, 1998; Brown, 1999; Jaenicke-Després *et al.*, 2003; Manen *et al.*, 2003). Valuable sequence information has also been obtained from DNA preserved in other plant tissues [e.g. herbarium specimens (Maunder *et al.*, 1999; Ristaino *et al.*, 2001; Saltonstall, 2002; DeCastro and Menale, 2004), wheat chaff used as ceiling insulation (Blatter *et al.*, 2002), and wood used in construction (Dumolin-Lapègue *et al.*, 1999)], ranging in age from 100 to 600 years.

The presence of useful DNA templates in ancient specimens highlights the unusual stability of the DNA molecule. While lysosomal or microbial nucleases rapidly degrade DNA into small fragments, models of degradation kinetics in the absence of nucleases predict that a fully hydrated DNA molecule will depolymerize into small fragments in about 10,000 years at room temperature (Lindahl, 1993). DNA primary (sequence) and secondary (helical) structures are sensitive to ionizing radiation and reactive oxygen species. Non-enzymatic degradation of DNA is initiated by cleavage of the N-glycosidic bond between the sugar backbone and the base. Further degradation, through oxidative reactions, leads to scission of the sugar–phosphate backbone, causing single- and double-strand breaks, chemical modification of bases and the crosslinking of carbonyl and amine groups (Lindahl, 1993; Britt, 1996; Halliwell and Gutteridge, 1999). Maillard reactions perpetuate degradation of DNA fragments into so-called advanced glycosylation endproducts (AGEs) (Britt, 1996; Halliwell and Gutteridge; 1999). Some researchers noted a black precipitate, indicative of severely oxidized products that might inhibit polymerase chain reactions (PCR), in extractions from herbarium specimens that were more than 60 years old (Savolainen *et al.*, 1995; Maunder *et al.*, 1999).

Aged specimens usually give low yields of low molecular weight DNA (Rogers and Bendich, 1985; Taylor and Swann, 1994; Savolainen *et al.*, 1995; Brown, 1999; Drábková *et al.*, 2002; Gugerli *et al.*, 2005). As the DNA template becomes progressively fragmented, it becomes increasingly difficult to amplify large molecular weight products through PCR (Pääbo *et al.*, 1989, 2004). Degraded fragments may also resist PCR amplification because primers hybridize poorly with modified bases or polymerase activity is blocked by base-free or cross-linked residues (Höss *et al.*, 1996; Vasan *et al.*, 1996; Poinar *et al.*, 1998; Halliwell and Gutteridge; 1999; Shirkey *et al.*, 2003; Threadgold and Brown, 2003). Many laboratories advocate special extraction procedures to increase yield or molecular weight, or to eliminate PCR inhibitors in deteriorated specimens (Pääbo *et al.*, 1989, 2004; Savolainen *et al.*, 1995; Poinar *et al.*, 1998; Drábková *et al.*, 2002).

The purpose of this paper is to examine the quality of DNA extracted from aged, but not ancient, seeds. Most gene banks maintain viable seeds that are regenerated when germination decreases to an unacceptable level. We wished to determine whether DNA in non-viable seed samples is of sufficiently high quality to justify retaining them in a DNA bank. A common method was used to extract DNA from seeds that were about 135, 70 and 40 or less years old, and DNA quality was assessed to illustrate that dead seeds, like herbarium leaf samples, can provide useful material for DNA banks

Materials and methods

Plant materials, provenance and viability assessments

One set of seeds used in this study was found in an attic in Madison, Georgia, USA, wrapped in letters and newspapers dating from 1863 through 1878. The undisturbed context in which the seeds were recovered suggested that they dated to the American Civil War era, and were about 135 years old. The seeds were archived in the Southern Seed Legacy Project at the University of Georgia, Athens, USA (http://www.uga.edu/~ebl/sslp/), and a few seeds were sent to our facility for testing [personal communications with Marshall Williams, Morgan County Archives (http://www.rootsweb.com/~usgenweb/ga/morgan.htm, also http://ftp.rootsweb.com/pub/usgenweb/ga/schley/newspapers/1907.txt) and Robert Rhoades, Department of Anthropology, University of Georgia; also Shearer, 2001]. Macroscopic features of these seeds were intact, and species were identified as *Cucurbita pepo* (pumpkin or squash), *Tragopogon porrifolius* (salsify), *Lagenaria siceraria* (calabash or bottle gourd) and *Cucumis* spp. (melon or cucumber) (see Fig. 1). These seeds would have been exposed to average yearly temperatures of 17°C, with extremes of 41°C in July 1980 and −22°C in January of 1985, and average relative humidity of 70% (Southeast Regional Climate Center, http://water.dnr.state.sc.us/climate/sercc/index.html).

A second set of seeds of *C. pepo* (A-265 and A-265-4 S1 Italian of Naples Vegetable Marrow, harvested in

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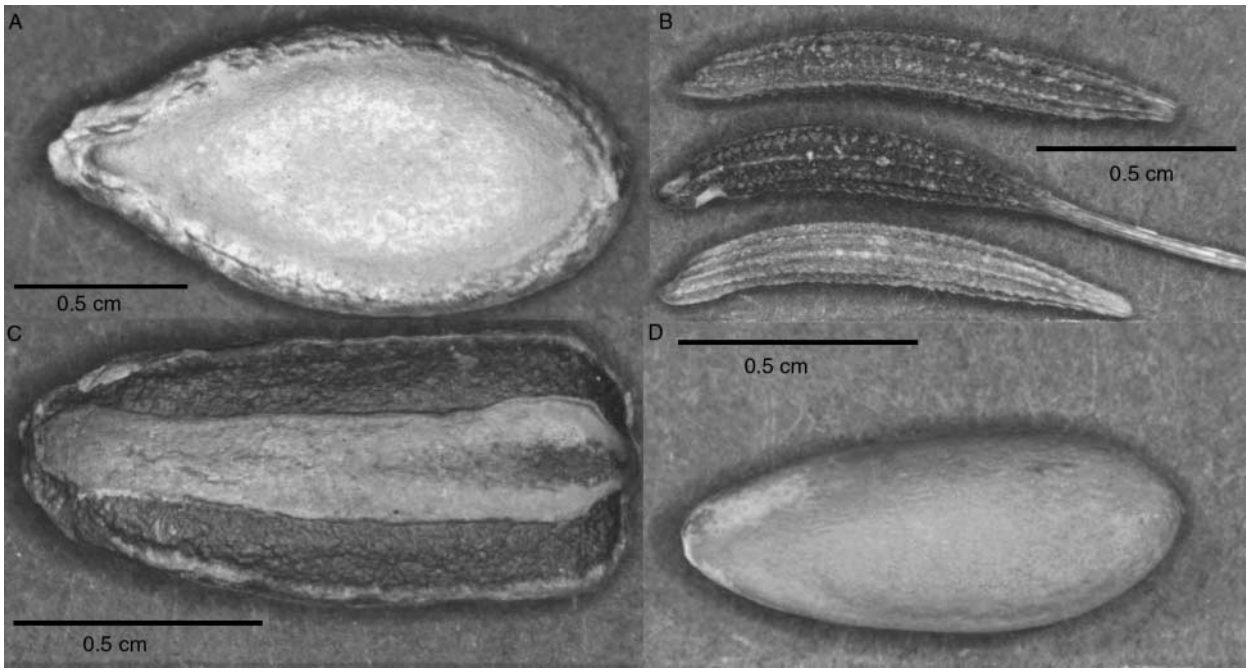

Figure 1. Seeds presumed to be about 135 years old that were found in an attic in Madison, Georgia, USA. Species were identified based on morphological features as (A) *Cucurbita pepo* (pumpkin or squash); (B) *Tragopogon porrifolius* (salsify); (C) *Lagenaria siceraria* (calabash or bottle gourd); and (D) *Cucumis* spp. (melon or cucumber).

1935 and 1937, respectively) were available from a collection of breeding lines and released cultivars. These seeds were stored in paper bags under laboratory room conditions in Athens, Georgia, USA (room temperature and similar humidity to the *c.* 135-year-old seeds) until 1965, when they were transferred to Fort Collins, Colorado, USA, and stored in the original paper bags at 5°C. In 1978, the temperature of the storage room was reduced to −18°C. The 1935 sample had visible fungal contamination on the testa, and 'heavy fungus' was noted during a 1984 germination assay. Although no evidence of fungi was recorded for the 1937 sample, the dry seeds appeared to be infested as well.

Seeds from the NCGRP (National Center for Genetic Resources Preservation) collection were also included for comparison (see Table 1). Accessions of *C. pepo* (PI 5207), *T. porrifolius* (PI 6405) and *C. melo* (PI 5658) were harvested in 1961. These seeds contained 0.05 g g^{-1} water and were stored at 5°C until 1978 and then at −18°C. An accession of *L. siceraria* with similar age and viability was not available, so we selected PI 235630, which was harvested in 1988, but had low viability. Accessions of *C. pepo* (PI 180768 and 425736), *C. melo* (PI 408245) and *C. sativus* (PI 247566) were harvested in 2001 or 2002, and were used as freshly harvested, high-quality controls. Freshly harvested seeds of *L. siceraria* and *T. porrifolius* were not available, so we used PI 309593 (harvested in 1995) and PI 97503 (harvested in 1978), respectively, and purchased varieties that were packaged for 2002 planting (Stokes Seeds, Fredonia, New York, USA). These lots were all recently tested for viability and were of high quality. A 1999 harvested accession of *C. sativus* (PI 386406) that had low germination was also included.

Seeds were assayed for viability under conditions recommended by AOSA (2003) for each species. Fifty seeds were used for most accessions, but only 3–5 seeds from the *c.* 135-year-old samples were available. Seeds were prehydrated with water vapour overnight to avoid imbibitional injury. Viability of the *c.* 135-year-old seeds was also evaluated by tetrazolium staining of 3–4 seeds of each species, if there was sufficient turgor after the first 24 h of imbibition (Peters, 2000).

DNA extraction and characterization

DNA integrity was assessed from individual seeds of each species and age class. Seed coats were removed from seeds after a preliminary 10-min soak in 10% bleach solution containing a drop of Tween 20. Whole embryos, or separated embryonic axes and cotyledons (*C. pepo* and *L. siceraria* only), were ground into a powder in the presence of liquid nitrogen, and DNA was extracted and purified using the DNeasy Mini Kit

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Table 1. Physical traits, viability and DNA quality and yield of seeds ranging in age from 1 to *c.* 135 years. Symbols indicate that the trait was strongly observed (+) or not observed (−) in all samples from the accession. Separate observations of the cotyledon (c) or embryonic axis (a) are indicated

									Total DNA extracted (μg/seed)[2]			Quality of DNA detected		
													PCR product	
Genus	Species	Accession	Harvest year	Seed mass (mg)	Seed length (cm)	Initial % germination	No. of seeds for DNA analysis	Current % germination	Embryo	Cotyledon[3]	Axis[3]	~ 10 kbp band	*ITS*	*rbcL*
Tragopogon	*porrifolius*	SSLP[1]	~ 1870	6.6 (3.8)	1.3 (0.8)	unknown	2	0	0			−	−	−
		6405	1961	12.1 (4.1)	1.2 (0.2)	98	1	64	2.3			+	+	+
		97503	1978	14.4 (1.5)	1.3 (0.1)	94	1	94	3.6			+	+	+
Cucumis	sp.	SSLP	~ 1863	Nd	1.0 (0.1)	unknown	3	0	<0.005			−	+ (faint)	+ (faint)
Cucumis	*melo*	5658	1961	21.0 (0.6)	0.8 (0.0)	77	1	32	17[a]			+	+	+
		408245	2001	20.7 (3.2)	0.8 (0.0)	100	1	100	12[a]			+	+	+
Cucumis	*sativus*	386406	1999	15.9 (2.8)	1.0 (0.1)	28	1	24	14[a]			+	+	+
		247566	2001	35.3 (1.1)	1.1 (0.0)	100	1	100	18[a]			+	+	+
Lagenaria	*siceraria*	SSLP	~ 1870	98.8 (6.3)	1.1 (0.1)	unknown	6	0	<0.005[a]	4.3[b] (4.2)	3.2[b] (5.0)	−	+ (faint)	+ (c) + (a)
		235630	1988	210.9 (34.7)	1.6 (0.1)	20	1	28	5[a]			+	+	+
		309593	1995	220.7 (49.3)	1.9 (0.3)	98	1	98	10[a]			+	+	+
Cucurbita	*pepo*	SSLP	~ 1870	103.1 (13.6)	1.7 (0.1)	unknown	7	0	<0.005[a]	2.4[b] (1.5)	5.3[b] (2.2)	−	− (c) + (a)	+ (c) + (a)
		A-265	1935	180.0 (50.1)	1.8 (0.1)	unknown	16	0	12.6[b] (5.1)	4.1[b] (2.3)	1.4[b] (1.0)	Faint	+	+ (c) + (a)
		A-265-4	1937	184.0 (19.0)	1.8 (0.1)	unknown	16	0	3.4[b] (1.5)	2.3[b] (1.7)	1.4[b] (1.0)	Faint	+	+ (c) + (a)
		5207	1961	138.4 (56.6)	1.4 (0.2)	80	1	32	20[a]			+	Nd	+
		180768	2001	111.3 (5.5)	1.2 (0.0)	98	1	98	4[a]			+	Nd	+
		425736	2002	42.8 (8.2)	1.1 (0.1)	99	16	99	4.4 (5.3)	1.9[b] (1.5)	2.3[b] (1.0)	+	+	+(c) + (a)

[1] Southern Seed Legacy Project.

[2] DNA concentration calculated from A_{260} measurements. The superscripts a and b represent separate extractions, with greater care given to exclude the lipid interface from the supernatant in method b.

[3] Blank entries indicate that seeds were not dissected.

Values in parentheses indicate the standard deviation of the mean of 4–8 samples.

Nd, not determined.

according to the manufacturer's instructions (Qaigen, Valencia, California, USA). DNA concentration was measured using absorbance at 260 nm. DNA extracts were stored at −80°C until used. Amplification of ancient DNA is sometimes improved when DNA is extracted in the presence of *N*-phenacylthiazolium bromide (PTB), which cleaves the glycosylation bonds of Maillard products that block polymerase activity (Vasan *et al.*, 1996; Poinar *et al.*, 1998; Shirkey *et al.*, 2003). To test this possibility, we had DNA from *c.* 135-year-old seeds of *C. pepo* and *L. siceraria* extracted commercially using the specialized techniques (TraceGenetics LLC, Davis, California, USA).

Electrophoresis was used to visualize the variation in fragment sizes of DNA extracted from seeds. About 15 μl of extracted DNA from each sample was mixed with 3 μl 6 × loading dye (Promega, Madison, Wisconsin, USA) and loaded on to a 1% agarose 1 × Tris, Acetak, EDTA (TAE) gel. Electrophoresis was carried out at 70 V for 1.5 h. Gels were stained for 10 min with ethidium bromide, and examined with ultraviolet light using Gel Pro Analyzer software 3.0 (Media Cybernetics, Silver Spring, Maryland, USA). Fragment size was estimated by comparison with a 1 kbp ladder (Promega, Madison, Wisconsin, USA).

DNA extracted from seeds was amplified using the primers ITS1 and ITS4, which are specific for a 650 bp segment coding for the internal transcribed spacer region (*ITS*) of the nuclear ribosomal DNA repeat (Baldwin *et al.*, 1995). We also used primers that targeted a 487 bp segment of *rbcL*: 1352R (CTTCACAAGCAGCAGCTAGTTCAGGACTCC, provided by R.G. Olmstead, University of Washington, Seattle, Washington, USA) and Z895 (GCAGTTATTGATAGACAGAAAATCATGGT, G. Zurawski, DNAX, Palo Alto, California, USA). These loci were targeted because (1) there are multiple copies per cell, increasing the probability of finding intact molecules in severely degraded samples; and (2) they can often be used to identify species (e.g. efforts by the Consortium for the Barcode of Life, www.barcoding.si.edu). DNA amplifications were performed in either 10 or 25 μl total volume, containing 10 mM Tris HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate (dNTP), 2.5 μM primers, 1.5 units of *Taq* DNA polymerase (Promega) and 0.2–2 μl total extracted DNA as template (larger volumes were used for the *c.* 135-year-old seeds). PCR was carried out in an MJ Research PTC-200 thermocycler (Waltham, Massachusetts, USA) using the following cycling profile: 95°C for 1 cycle 5 min; 35 cycles 95°C for 30 s, 52°C for 30 s, 72°C for 30 s; and a final elongation step at 72°C for 1 min. Standard precautions were taken to prevent and monitor contamination of DNA extractions and PCR reagents, including extraction and water blanks, none of which yielded PCR products. PCR products were separated by gel electrophoresis in 2% agarose TAE gels. DNA bands were visualized by ethidium bromide staining. Molecular weights were determined by comparison with the 100 bp ladder (Promega).

PCR products were cloned using the TOPO® TA Cloning kit (Invitrogen, Carlsbad, California, USA). Positive colonies were identified by PCR and sequenced (LiCor IR^2 Sequencer, Lincoln, Nebraska, USA). Sequences were edited using Sequencher 4.1 software (Gene Codes Corporation, Ann Arbor, Michigan, USA). Edited sequences were compared to entries in the GenBank Nucleotide Sequence Database using the BLASTn program (NCBI, Bethesda, Maryland, USA).

Results

The *c.* 135-year-old seeds were morphologically similar to modern-day cultivars (Fig. 1, Table 1), although they appeared slightly browner and floury (indicative of microbial infestation). Seeds were extremely brittle and difficult to dissect. The cotyledons of the cucurbit seeds were light orange and waxy (compared to cream coloured in fresh seeds) with pieces missing. Fungal mycelia were evident between the cotyledons. The integument layers lacked the green colour evident in fresh seeds. Despite similar lengths, seeds of *C. pepo* and *T. porrifolius* weighed less than their modern-day counterparts (Table 1) (AOSA, 2003). Old seeds of *L. siceraria* weighed less and were shorter than modern-day counterparts. The 135-year-old seeds did not germinate and did not stain positively with tetrazolium, although a portion of the seeds from all species except *T. porrifolius* became turgid upon imbibition (data not shown). Germination records for the *C. pepo* seeds harvested in 1935 and 1937 showed 0% viability in 1984 (Table 1).

Viability time courses for NCGRP accessions harvested in the 1960s through 1980s are well documented and follow three patterns: initially high germination that decreased during 44 years of storage (PI 5207, 5658, 6405, corresponding to *C. pepo*, *C. melo* and *T. porrifolius*), initially low germination that stayed constant for 15 years (PI 235630 corresponding to *L. siceraria)* and initially high germination that remained constant for 25 years (PI 97503 corresponding to *T. porrifolius*) (Table 1). Seeds harvested more recently have high germination percentages, with the exception of accession PI 386406 of *C. sativus* (Table 1).

The quantity of DNA extracted from individual seeds was estimated by measuring the absorbance at 260 nm. DNA yield varied from below the limits of detection (< 0.005 μg) for the *c.* 135-year-old seeds to 20 μg per seed (Table 1). Because of poor initial yields from *c.* 135-year-old *C. pepo* and *L. siceraria* seeds,

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a second extraction was attempted. When extra care was used in grinding seeds, and excess storage lipids present in the crude lysate were avoided, more DNA was recovered. DNA yields from cotyledons and embryonic axes of *C. pepo* and *L. siceraria* were similar despite the difference in mass between the two organs (Table 1). The DNA yields from samples of *c*. 135-year-old seeds of *C. pepo* and *L. siceraria*, extracted in the presence of PTB, were below our detection limit (data not shown).

Genomic DNA from seeds in the NCGRP collection (harvested in or after 1961) was primarily of high molecular weight, with the band of greatest intensity appearing above 10.5 kb (representative gel given in Fig. 2A). There were no obvious differences in electrophoretic pattern between these seeds and those packaged for planting in 2002, even though there were differences in viability (see Table 1 for percentage germination). High molecular weight DNA was also successfully extracted from *C. pepo*

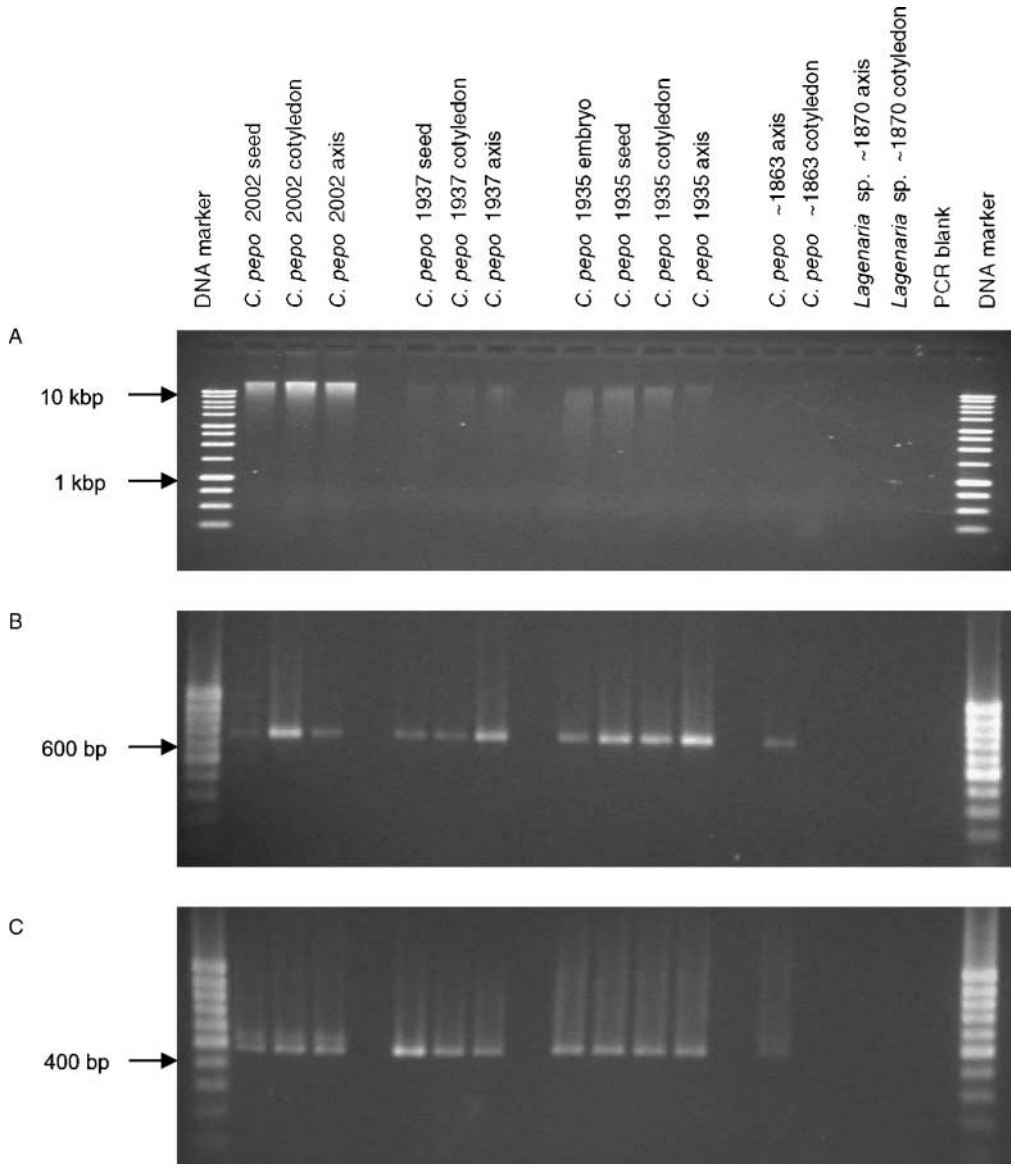

Figure 2. Agarose gel images of genomic DNA (A) and polymerase chain reaction (PCR) amplification products using primers targeted to the internal transcribed spacer (*ITS*) region (B) and *rbcL* (C); 15 µl of genomic DNA extract were loaded (concentrations indicated in Table 1). DNA from ~135-year-old samples was difficult to visualize using ethidium bromide, regardless of the volume of DNA used.

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seeds harvested in 1935 and 1937 (Fig. 2A). However, DNA extracted from the *c.* 135-year-old seeds could not be seen on ethidium bromide-stained gels (Fig. 2A). Loading larger quantities of DNA on to the gels did not improve detection (data not shown). Densitometer scans of the gels revealed a very slight increase in fluorescence at molecular weights between 0.75 and 3 kb (data not shown).

The *ITS* region (Fig. 2B) and *rbcL* (Fig. 2C) were consistently amplified when using DNA extracted from modern samples (harvested in or after 1935) (Table 1). Some PCR amplifications that used DNA from the *c.* 135-year-old seeds resulted in products of the expected molecular weight (Fig. 2B, C; Table 1). No PCR products were obtained from the commercially prepared DNA samples from the *c.* 135-year-old seeds (data not shown).

PCR products from seed samples were cloned and sequenced to confirm their identity. DNA segments from the *ITS* region and chloroplast *rbcL* gene from modern seeds of *C. melo* and *C. pepo* were identical to their counterparts in the GenBank database (sequences for *C. sativus*, but not *C. melo*, are listed in GenBank). As of December 2005, there were no sequences available for *Lagenaria* sp. in GenBank. The *ITS* clone from the 1995 *L. siceraria* accession (309593) was 100% identical to *Cucumis globosus*, and the *rbcL* clone from this accession was 98% identical to *Coccinia adoensis*, *Cucumis sativus* and *Corallocarpus bainesii* (all species within *Cucurbitaceae*). Chloroplast *rbcL* sequences were obtained from two of the *c.* 135-year-old *Cucumis* seeds, and were identical to *Cucumis* sp. However, the *ITS* region amplified from these seeds appears to have been derived from the fungus *Aspergillus penicillioides*. Sequences from *c.* 135-year-old *C. pepo* seeds (DNA from all four seeds sequenced) and *L. siceraria* (three of four sequenced) were 95–100% identical to *C. pepo* chloroplast *rbcL*. *ITS* sequences from *c.* 135-year-old *L. siceraria* seeds were 100% identical to *Aspergillus*.

Discussion

We have shown that DNA can be extracted from severely deteriorated seeds using standard techniques, and that amplification products of relatively high molecular weight can be retrieved despite low yields of nucleic acids. The yield and quality of DNA extracted from stored seeds is similar to DNA extracted from herbarium leaf tissues (e.g. Savolainen *et al.*, 1995). Our results demonstrate that the utility of banked seeds can be extended beyond their traditional lifespan by considering non-viable seeds to be vessels for the storage of DNA.

DNA yield and molecular weight are dependent on the initial condition of the sample and storage conditions that prevent nuclease activity. Senescent leaf samples consistently provide poor-quality DNA (Rogers and Bendich, 1985; Pyle and Adams, 1989; Taylor and Swann, 1994; Savolainen *et al.*, 1995; Drábková *et al.*, 2002). We noted that the amount of DNA initially retrieved from fresh leaves is sometimes greater than the amount extracted from fresh seeds or cotyledons (based on microgram nucleic acid per milligram tissue).

Yield declines with storage time in both leaves and seeds (Table 1; Rogers and Bendich, 1985; Pyle and Adams, 1989; Ristaino *et al.*, 2001; Drábková *et al.*, 2002). DNA from badly charred seeds was often of higher quality than DNA from specimens that were morphologically well-preserved (Goloubinoff *et al.*, 1993; Brown, 1999; Threadgold and Brown, 2003). It is possible that charred seeds have been sterilized by heat, and so the initial and dramatic degradation of DNA at high temperatures may be balanced in the long run by the inhibition of microbial degradation. Inability to detect DNA on ethidium bromide-stained gels corresponded to a reduction in DNA from 115 ng seed^{-1} to 30 ng seed^{-1} in wheat (Threadgold and Brown, 2003). Poor visualization of electrophoresed DNA from ancient samples is a common problem since DNA degrades into a range of fragment sizes, and good staining requires abundant DNA at a particular molecular weight (e.g. Allaby *et al.*, 1994; Dumolin-Lapègue *et al.*, 1999). Visualization can be improved by using commercially available stains that are more sensitive than ethidium bromide.

Microbial effects on DNA stability underscore the importance of preservation conditions that limit microfloral proliferation. Amplification of *Aspergillus* spp. DNA sequences indicates that the *c.* 135-year-old seeds were contaminated. Highly fragmented DNA (Fig. 2) and preferential amplification of fungal DNA attest to the damaging effects of degrading microflora, as well as the increased risk of inadvertently amplifying DNA from a contaminating species. When universal primers are used, precautions are needed to validate the source organism of the template (e.g. Cooper and Poinar, 2000). When the identity of material is known, primers specific for the species are more appropriate.

To provide a broader context for the rate of DNA deterioration, we compiled published data on fragment and PCR product size from a variety of sources that used archived or ancient plant specimens for DNA analyses (Fig. 3). In older samples, DNA is more fragmented (left *y*-axis, solid symbols), and success of amplification depends on product size (right *y*-axis, open symbols). Data points were fitted to an exponential equation, and the time coefficient to describe deterioration rate was calculated for both seed and leaf tissues (Fig. 3A and B, respectively). Time coefficients for fragmentation (3.1 ± 0.4 and

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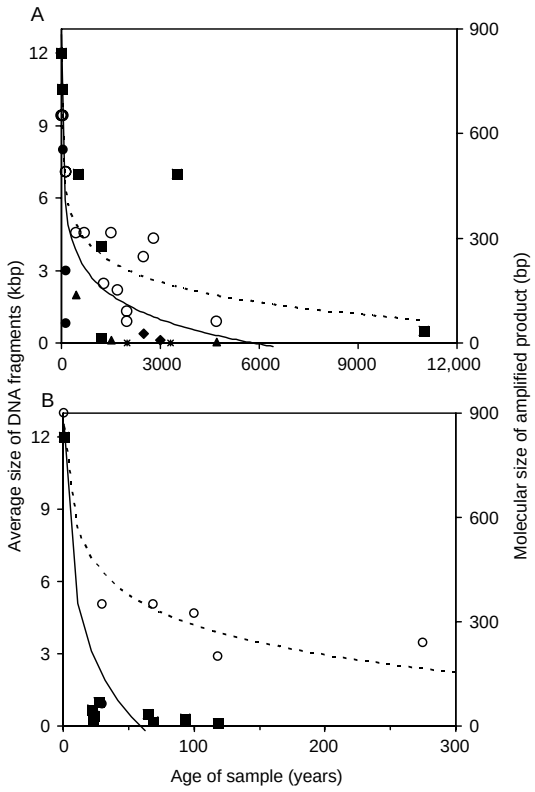

Figure 3. A compilation of reported data on the relationship between sample age and quality of extracted DNA from (A) seeds and (B) herbarium leaf samples. Quality was assessed by the average size of genomic DNA fragments (solid symbols) and the maximum documented length of amplification products (open symbols). Data are fitted to logarithmic curves as an example of the time course for DNA degradation in the two specimen types. Calculated intercepts for the fragment size are about 13 kbp, and for amplification products are about 850 bp for both seeds and leaf tissues. The time coefficients for fragmentation (solid curves) are -1.67 ($r^2 = 0.74$) and -3.07 ($r^2 = 0.87$) for seeds and leaf tissues, respectively. The time coefficients for size of amplification product (dashed curves) are -81 ($r^2 = 0.75$) and -124 ($r^2 = 0.93$) for seeds and leaf tissues, respectively. In (A), data for fragment size (solid symbols) are taken from densitometer scans from Fig. 2 (circles); Rogers and Bendich, 1985 (squares); Rollo *et al.*, 1991 (asterisks); Goloubinoff *et al.*, 1993 (triangles); and Allaby *et al.*, 1994, 1997 (diamonds). Data for amplification products (open circles) are from Table 1; Rollo *et al.*, 1991; Goloubinoff *et al.*, 1993; O'Donoghue *et al.*, 1996; Deakin *et al.*, 1998; Freitas *et al.*, 2003; Jaenicke-Desprès *et al.*, 2003; and Manen *et al.*, 2003. In (B), data for fragment sizes (closed symbols) are from Rogers and Bendich, 1985 (squares) and Savolainen *et al.*, 1995 (circles). Data for amplification products (open circles) are from Savolainen *et al.*, 1995; Ristaino *et al.*, 2001; Blatter *et al.*, 2002; Drábková *et al.*, 2002; Saltonstall, 2002 (verified by personal communication); and De Castro and Menale, 2004. In (A), the data point from 11,000-year-old seeds was not used in the analysis, but is shown for interest.

1.7 ± 0.3 for leaf and seed data, respectively) and for PCR product size (124 ± 16 and 81 ± 13 for leaf and seed data, respectively) are greater in leaf compared to seed tissues, suggesting that DNA degradation is faster in leaf samples according to both measures of DNA integrity. DNA from leaf specimens that were stored mostly in herbaria degraded into small fragments within 100 years, and the model predicts that PCR product size will decrease to less than 300 bp after 94 years (Fig. 3B). The low r^2 values of the fitted curve for seed data attest to greater variability of degradation rates in seeds compared to leaves, likely because environments where ancient seeds are found are not highly controlled. According to the relationships depicted in Fig. 3A, DNA in seeds degrades into small fragments over thousands of years, and PCR product size will decrease to less than 300 bp after 675 years. These conclusions do not change substantially if regression models use only data from specimens ≤ 150 years old. The kinetics of DNA degradation in seeds are consistent with models by Lindahl (1993), who predicted that DNA stored at room temperature would be degraded into small fragments in 10,000 years. DNA degrades to a similar level within 5 h and 30 min at 150 and 200°C, respectively, which is consistent with an involvement of Maillard-type reactions (Threadgold and Brown, 2003).

This survey of the literature suggests that DNA degrades more rapidly in leaf compared to seed tissues; however, more rigorous side-by-side analyses of DNA fragment length and amplification success within leaf and seed tissue harvested at the same time and stored in the same way are required. Nonetheless, our study shows that embryonic axes of seeds may be ideal sources of DNA because the high density of cells provides multiple copies of DNA templates, and the seed outer coverings may protect DNA from degenerative processes (Gugerli *et al.*, 2005).

Conclusions

Most species of seeds survive for decades or more under the refrigerated conditions common in gene banks (Walters *et al.*, 2005). DNA extracted from these seeds is expected to have high molecular weight and to provide a suitable template for PCR amplification. Seeds that have lost viability may still be valuable as vessels of genetic information. We have shown that large molecular weight fragments can be amplified in 70- and 135-year-old seeds that were stored under ambient conditions, even though significant deterioration of DNA quality was evident. This highlights the potential utility of post-mortem DNA in seed samples for identifying species, determining historical distributions of populations, documenting parental lines of improved germplasm, and inferring evolution of

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genome structure. The flexibility for bulking procedures and the additional morphological detail provided by seed structure enhance the usefulness of seeds as vessels for DNA. Although not all non-viable seeds will be useful, gene bank operators can consider the availability of high-quality DNA as an additional benefit of seed banks.

Acknowledgements

We greatly appreciate the donation of the heirloom seeds by Drs Robert Rhoades and Virginia Nazarea (Department of Anthropology, University of Georgia), and inspiration for the research provided by Dr Henry Shands (USDA-ARS National Center for Genetic Resources Preservation). We also thank Lana Wheeler for all her efforts in retrieving information and seeds from the NCGRP collection.

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Received 24 August 2005
accepted after revision 29 May 2006