Seed Science Research (2009) **19**, 125–135 doi:10.1017/S0960258509990055

DNA preservation and utility of a historic seed collection

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Abstract

Historic collections of biological material are important genetic resources for taxonomic, evolutionary and historical research. In this paper we describe a seed collection dating from 1862 to 1918 maintained at the Swedish Museum of Cultural History. The collection contains over 3000 well-documented seed samples of various agricultural crops, mostly cereals. A subset of 100 samples divided over ten species frequently represented in the collection and a range of ages were tested for germinability and DNA preservation. None of these accessions were found to contain viable seeds. DNA extracted from the seeds was degraded, but the amount of degradation varied between species. DNA quality was evaluated by yield, fragment size and size of amplification product. Quality was highest for DNA extracted from *Pisum sativum* and *Vicia sativa*. DNA extracted from *Brassica napus*, *Beta vulgaris* and *Trifolium pratense* was more fragmented, and DNA extracted from *Triticum aestivum*, *Secale sereale*, *Hordeum vulgare*, *Avena sativa* and *Phleum pratense* was most degraded. Polymerase chain reaction (PCR) amplification of ribosomal DNA fragments of up to 700 bp was permitted for most samples in all species. To test whether single-copy nuclear genes could be amplified from the extracted DNA, microsatellite markers were used on the *Pisum sativum* and *Hordeum vulgare* samples. Polymorphisms of microsatellite markers were detected between samples for both species. The results show that the 19th-century seed collection can be utilized to infer genetic relationships among obsolete cultivars as well as for other types of genetic research based on sequence or marker analysis.

Keywords: aged seed, DNA, genebank, microsatellites, plant genetic resources, stability

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Introduction

The evolution of crop plants can be described as a two-step process, where the first step is the domestication of wild plants and the second step the improvement of primitive landraces into cultivars by plant breeding. Both these two steps are associated with losses of genetic diversity, known as the domestication and improvement bottleneck (Tanksley and McCouch, 1997). Recent research, involving selection screens, quantitative trait loci (QTL) mapping and allele mining, has begun to reveal the molecular genetics behind the evolution of crop plants (reviewed by Doebley *et al.*, 2006). Cloning the superior alleles that contributed to the domestication and improvement process has been of interest not only to the research community but also to plant breeding companies. Obviously, one key to future crops lies in understanding the evolutionary history of crops from the past.

One obstacle for conducting this type of survey can be the lack of wild and unimproved plant material. Since the introduction of improved cultivars around 1900, landraces and obsolete cultivars have rapidly become extinct (FAO, 1997). In northern Europe only a small fraction of the landraces that were the basis for agriculture for thousands of years has been preserved in gene banks. Furthermore, landraces and obsolete cultivars maintained by breeding companies and gene banks risk losing genetic integrity during regenerations (Steiner *et al.*, 1997; Börner *et al.*, 2000; Chebotar *et al.*, 2002).

As a supplement to living material, specimens maintained in museums, for example, can be used for molecular genetic analyses. Although research on aged and ancient DNA has focused very much on humans and other mammals, attention to plants has increased lately (reviewed by Gugerli *et al.*, 2005). Seeds are a common plant material for the analysis of aged and ancient DNA, and DNA has been isolated and analysed successfully from seeds of up to 4000 years of age, from different species (Blatter *et al.*, 2002; Freitas *et al.*, 2003; Jaenicke-Després *et al.*, 2003; Manen *et al.*, 2003; Walters *et al.*, 2006; Lia *et al.*, 2007; Lister *et al.*, 2008). These seeds have been found in archaeological excavations or, for the more recent samples, in buildings or even herbaria.

https://doi.org/10.1017/S0960258509990055 Published online by Cambridge University Press

A severe constraint to the use of non-viable historical material is that information attached to the material, such as dating, cultivar name and growth location, is often vague or lacking (Jones *et al.*, 2008). Another problem lies in the number of individuals available to sample, which is often too small to gain an understanding of population genetic aspects of the crop. In contrast to archaeological and herbaria samples, seed collections are more likely to contain plant material from many individuals, as well as having associated information that increases the strength of conclusions that can be drawn. An additional benefit of seed collections in comparison to herbaria is that DNA seems to degenerate more slowly in seeds than in vegetative plant parts (Walters *et al.*, 2006; Lister *et al.*, 2008).

In Europe several herbaria and seed collections of crop plants from the 19th and early 20th century have been preserved (Jones *et al.*, 2008). Perhaps, best recognized is the Percival Collection of wheats from the 1920s stored at the Natural History Museum in London, UK (Morrison, 2001). Seed material from this collection has been utilized successfully to assess DNA preservation (Lister *et al.*, 2008). In the Museum of Vänersborg, Sweden, 1800 seed samples from an agricultural exhibition in 1880 are stored (Johansson *et al.*, 2003). Seeds from this collection have been used for amplified fragment length polymorphism (AFLP) analyses of a *Beta vulgaris* cultivar, where the aged seeds were compared with homonymous plants preserved 'on-farm', and it was concluded that the cultivated accession had changed dramatically (Poulsen *et al.*, 2007).

A very large 19th-century seed collection has been stored at the Swedish Museum of Cultural History since it was donated by the Royal Swedish Academy of Agriculture and Forestry (KSLA) in 1963. The collection contains several sub-collections of seeds from exhibitions, collection expeditions, seed testing and breeding institutes, as well as from test cultivations. The collection is used mostly for display and has not been analysed for its research potential. An inventory of the KSLA seed collection was made and sub-samples representing different species and ages were analysed for viability and DNA quality. Here, we show the results of this screen of the seed collection. Furthermore, we demonstrate that molecular markers, such as microsatellites, can be used to determine genetic relationships between the accessions found in the seed collection.

Materials and methods

Seed material

Newly harvested seeds had been stored in glass containers sealed with either glass or wooden lids or, most commonly, cork plugs (Fig. 1). The seed containers were stored at room temperature, except for brief periods at lower temperatures. The approximate water content of seeds was 7–10%. Sub-samples from 100 accessions representing ten species differing in age and origin were taken from the containers (see Table 2). Fresh seeds of each species, provided by SW Seed, Svalöv, Sweden or Runåberg Seeds, Spekeröd, Sweden (*Beta vulgaris* cv. Boltardy) were used as positive controls.

Germination tests

Germination tests were performed using two different methods. In a standard germination test, 25 (*Pisum sativum*, *Vica sativa* and *Beta vulgaris*) or 50 seeds

Figure 1. Examples of seed containers in the KSLA seed collection.

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(all other species) were planted in or on top of sterile sand (ISTA, 1999). Seeds were also placed on agar using modifications to a method especially developed for aged seed (Poulsen *et al.*, 2006). The agar method consisted of surface sterilizing 50 (*Pisum sativum*, *Vica sativa* and *Beta vulgaris*) or 100 seeds (all other species) in 70% ethanol for 30 s followed by 8 min in 1.5% sodium hypochlorite containing a few drops of Tween, then placing seeds on $\frac{1}{4}$ strength Murashige and Skoog (MS) medium (Duchefa, Haarlem, The Netherlands) solidified with 0.5% plant agar (Duchefa, Haarlem, The Netherlands). Germination assays were performed in a growth chamber at 20°C and 16 h light. Plates were inspected every second day for 50 d.

DNA extraction and analysis

DNA was isolated from 30–70 mg of dry seeds of each accession using the FastDNA® Spin Kit and the FastPrep® Instrument (Qbiogene Inc., California, USA). This procedure, where the seeds are ground in sealed individual tubes, allows rapid and efficient homogenization without sample-to-sample contamination risk. DNA was extracted from single seeds of cereals or one-quarter to one-half seeds for *Pisum sativum* and *Vicia sativa*. Unfortunately, insufficient DNA to be visible on gels was extracted from single individuals from the other species. Thus, one glomerule of *Beta vulgaris*, 10 seeds of *Brassica napus*, 20 seeds of *Trifolium pratense* and 50 seeds of *Phleum pratense* were used in each extraction. DNA was eluted in 100 µl of the supplied buffer. Negative controls were performed in parallel with each extraction series.

Fragment sizes of extracted DNA were estimated by banding patterns of 20 µl of extract run on 1% agarose 0.5 × TBE (Tris-borate-EDTA) gels. The size markers GeneRuler™ LowRange (Fermentas, Burlington, Canada) and Quick-Load™ 1 kb DNA ladder (New England Biolabs, Ipswich, Massachusetts, USA) were included as references. Gels were stained with ethidium bromide (EtBr) and examined with ultraviolet (UV) light. Gels were inspected by eye to identify the most abundant fragment sizes (with maximum fluorescence).

Polymerase chain reaction amplification

Amplification of fragments of different sizes was performed using different primer pairs: internal transcribed spacer (*ITS*) region of nuclear ribosomal DNA ITS1 + ITS4 resulting in a ~700 bp fragment, ITS1 + ITS2 resulting in a ~350 bp fragment (White *et al.*, 1990), and nuclear ribosomal large subunit DNA LR6 + LR17R resulting in a 109 bp fragment (http://www.biology.duke.edu/fungi/mycolab/primers.htm). Polymerase chain reaction (PCR) amplifications were performed in a 20 µl reaction volume comprised of 1 U *Taq* polymerase (Invitrogen, Carlsbad, California, USA), 1 × Invitrogen buffer, 1.5 mM $MgCl_2$, 0.2 µM each primer, 0.2 mM each dNTP and 0.5 µl of DNA extract. Reaction runs consisted of 3 min at 94°C (initial denaturation): 35 cycles each of 94°C for 30 s, 48–60°C for 30 s and 72°C for 30 s; and a final elongation step of 72°C for 5 min. Annealing temperatures were 56, 60 and 48°C, for primers ITS1 + ITS4, ITS1 + ITS2 and LR6 + LR17R, respectively. Amplification products were electrophoresed on 1.5–2.0% agarose 0.5 × TBE gels and visualized by EtBr staining and UV light. Two ~700 bp PCR products from aged samples of each species were sequenced (MWG, Germany). In each PCR run the extraction blank was used as a negative control.

Analysis with microsatellite markers

Microsatellite markers from six loci were amplified in *Pisum sativum* and *Hordeum vulgare* samples (see Table 3). The same DNA extracts as used previously (from one individual seed from each accession) were analysed. Procedures for *Pisum sativum* used semi-nested PCR (Loridon *et al.*, 2005). Each 20 µl reaction contained 1 U *Taq* polymerase (Invitrogen), 1 × Invitrogen buffer, 1.5 mM $MgCl_2$, 0.07 µM forward primer with M13-tail at the 5′ end, 0.2 µM fluorescently labelled M13 primer, 0.27 µM reverse primer, 0.2 mM each dNTP and 0.5 µl of DNA extract. Either FAM or HEX were used as fluorescent dyes.

Microsatellite amplification of *Hordeum vulgare* DNA extracts followed Ramsay *et al.* (2000). The forward primers were again modified with M13-tails. PCR amplification was run in two separate rounds, the first with modified forward primers and reverse primers, the second with a fluorescently labelled M13 forward primer (FAM or HEX) and the marker's reverse primer. PCRs were run in 20 µl reactions containing 0.5 U *Taq* polymerase (New England BioLabs), 1 × New England BioLabs ThermoPol buffer, 0.8 mM $MgCl_2$, 0.1 µM each of forward and reverse primers and 0.25 µM of each dNTP. For the first round of PCR 1 µl of total DNA template was used, and 1 µl of the first PCR reaction was used as the template for the second round of PCR.

Amplification products were analysed by capillary gel electrophoresis and confocal laser scanning on a MegaBACE 500 DNA Analysis System (Amersham Biosciences, Uppsala, Sweden) using a 400 bp ROX-labelled internal size marker for *Pisum sativum* samples and on a MegaBACE 1000 DNA Analysis System using a 350 bp ROX-labelled internal size marker for *Hordeum vulgare* samples. Sizing of fragments was performed using the software MegaBACE Fragment Profiler 1.2 (Amersham Biosciences).

https://doi.org/10.1017/S0960258509990055 Published online by Cambridge University Press

Population subdivision between two-row and six-row *Hordeum vulgare* accessions (#192, #288, #717, #762, #787, #1818 and #659, #737, #1584, #1822 and cv. Rolfi, respectively) was estimated using Wright's F_{ST} (Wright, 1951).

Results

The KSLA seed collection consists of 3393 accessions, mostly containing several hundred seeds each, representing 582 agronomically important species. About 96.5% of the accessions displayed excellent morphological preservation. The remaining samples, where the container or seal was broken, were completely or partly destroyed by mould or insects. The collection is comprised of several sub-collections (A–J): A, samples from test cultivations in the experimental field in Stockholm 1865–1894; B, a country-wide collection of samples from farmers' harvests in Sweden 1867; C, like B but from 1896; D, like B but from Finland 1882; E, seed from an exhibition in Italy 1873; F, seed from an exhibition in Philadelphia, USA 1876; G, seed collected by the first Swedish seed testing institute in Nydala, province of Halland and donated to the academy in 1877; H, seed for pharmaceutical use collected by the Karolinska Institutet in Africa and Asia from the 1850s and onwards, and donated to the academy in 1899; I, samples donated from the breeding company Vilmorin et Andrieux, France 1908; J, samples donated from the breeding company Svalöf, Sweden 1918. In addition to these major sub-collections several minor sub-collections and single samples are found. The distribution of the most frequently represented species by sub-collection is displayed in Table 1. The collection is comprised mostly of cereal grains (56%), but also has a smaller number of forage crops, vegetables, forest trees, other useful plants and important weeds. Most accessions are labelled with harvest year, name of cultivar (if applicable) and growth location.

To analyse viability and DNA preservation, sub-samples were taken from 100 accessions representing ten different species and a range of ages (Table 2). No seeds from the collection germinated, whereas fresh seed samples from the same species all germinated to over 90%. Thus, seeds in the collection of the ten tested species are most likely non-viable. DNA yields were typically between 100 and 300 ng mg^{-1} seed, with the highest yields from *Pisum sativum* and the lowest from *Beta vulgaris*. In fresh seed DNA yields were typically 3–5 times higher.

DNA isolated from fresh seed samples was primarily of high molecular weight, with the greatest intensity of fragments above 10 kb (Fig. 2a). In contrast, DNA isolated from the aged seed showed various

Table 1. Distribution of accessions by species in the major sub-collection. Letters refer to the sub-collections (see Results). Only sub-collections with more than 40 accessions and species with more than ten accessions are individually presented. Other species and sub-collections are summarized

	Sub-collection											
Species	A	B	C	D	E	F	G	H	I	J	Other	Total
Triticum aestivum	139	0	110	11	19	6	96	5	53	34	71	544
Avena sativa	54	11	287	14	3	3	51	3	15	18	42	501
Hordeum vulgare	69	15	194	19		3	52	2	12	11	39	419
Secale cereale	34	24	176	26	1	2	35	1	8	14	3	324
Pisum sativum	1	16	129	12		2	20	1		3	27	211
Vicia sativa			54				14				7	75
Phaseolus vulgaris				2	4	2	12	16			11	47
Vicia faba			14	1	6	1	6	1		2	6	37
Beta vulgaris			2			6	10	1		1	12	32
Brassica napus		1	10				12				6	29
Brassica rapa			1			2	21				3	27
Lens culinaris						6	13	4			2	25
Zea mays					2	3	15	1			4	25
Phleum pratense		1	13	7		1	1					23
Oryza sativa						7	2	12			1	22
Trifolium pratense		1	10	3			5				1	20
Linum usitatissimum			2		1	1	10				5	19
Brassica oleracea			6				8			1		15
Sorghum bicolor						2	7	6				15
Panicum milliaceum					3		7	2			1	13
Allium cepa						2	8					10
Other species	7	5	19	7	9	72	637	103		3	101	960
Total	304	74	1027	102	48	121	1042	158	88	87	342	3393

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Table 2. Accessions from the seed collection and fresh reference samples tested for DNA fragment size and PCR amplification. Accession number (Acc. #) refers to the seed collection inventory number in the Swedish Museum of Cultural History. Nd = not detected; * indicates that the product was sequenced to confirm species identity; + and − indicate presence or not, respectively, of amplification product using ITS and LR primer sequences with the indicated fragment length and (+) or (−) indicates amplification results after DNA extracts were diluted

						PCR amplification		
Acc. #	Species	Cultivar name	Growth location	Harvest year	DNA fragment size (bp)	109 bp	350 bp	700 bp
132	*Triticum aestivum*	Rouge d'Altkirch	Paris (F)	1908	Nd	−	−	−
134	*Triticum aestivum*	Browick	Paris (F)	1908	Nd	−	−	−
183	*Triticum aestivum*	Hvitaxigt sammetskubb	Stockholm (SWE)	1891	250	+	+	+
257	*Triticum aestivum*	Kubbvete	Meldola (FIN)	1882	300	+	+	+
527	*Triticum aestivum*	Kjulsta stamvete	Kjulsta (SWE)	1896	250	+	+	+
531	*Triticum aestivum*	Landthvete	Hagby (SWE)	1896	150	+	−	−
1763	*Triticum aestivum*	Hicklings prolific	Stockholm (SWE)	1865	200	+	+	−
1906	*Triticum aestivum*	Saxons	Stockholm (SWE)	1897	300	+	+	+*
3210	*Triticum aestivum*	Blue Sterne	Kansas (US)	1876	300	+	+	+*
3234	*Triticum aestivum*	Grano Saragolla	(IT)	1873	200	+	+	−
	Triticum aestivum	SW Kosack	Svalöv (SWE)	2006	>10,000	+	+	+
77	*Secale cereale*	d'Hiver de Schlanstedt	Paris (F)	1908	Nd	−	−	−
198	*Secale cereale*	Christensens Riesen	Stockholm (SWE)	1892	300	+	+	+*
252	*Secale cereale*	Local cultivar	Tervik Perno (FIN)	1882	300	+	+	+
258	*Secale cereale*	Local cultivar	Perheniemi (FIN)	1882	200	+	+	+
1753	*Secale cereale*	Probsteier	Stockholm (SWE)	1865	300	+	+	+
1755	*Secale cereale*	Unknown	(BLR)	1873	100	+	−	−
3208	*Secale cereale*	Local cultivar	Leksand (SWE)	1867	200	+	+	−
3329	*Secale cereale*	Uplandsråg	Gärdslösa (SWE)	1896	300	+	+	+
3337	*Secale cereale*	Svenskråg	Örebro (SWE)	1896	150	+	+	−
3339	*Secale cereale*	Tufråg	Öjebyn (SWE)	1896	300	+	+	+*
	Secale cereale	SW Amilo	Svalöv (SWE)	2006	>10,000	+	+	+
192	*Hordeum vulgare*	Skånskt Chevalier	Stockholm (SWE)	1889	200	+	+	+*
288	*Hordeum vulgare*	Local cultivar	Ingeris (FIN)	1882	200	+	+	+
659	*Hordeum vulgare*	Local cultivar	Geråg (SWE)	1896	150	+	+	−
717	*Hordeum vulgare*	Golden Thorpe	Kristineberg (SWE)	1896	150	+	+	−
737	*Hordeum vulgare*	Local cultivar	Tjös (SWE)	1896	150	+	+	−
762	*Hordeum vulgare*	Landtkorn	Angerum (SWE)	1896	150	+	+	+*
787	*Hordeum vulgare*	Ölandskorn	Gärdslösa (SWE)	1896	150	+	+	+
1584	*Hordeum vulgare*	Local cultivar	Kilafors (SWE)	1867	150	+	+	−
1818	*Hordeum vulgare*	Berwick	Stockholm (SWE)	1865	150	+	+	+
1822	*Hordeum vulgare*	Hauben oben Dreizack	Stockholm (SWE)	1865	150	+	+	+
	Hordeum vulgare	Rolfi	Svalöv (SWE)	2006	>10,000	+	+	+
71	*Avena sativa*	Von Lochows Gelbhafer	Svalöv (SWE)	1918	400	+	+	+*
231	*Avena sativa*	Schotticher August	Stockholm (SWE)	1894	300	+	+	+
269	*Avena sativa*	Local cultivar	Rivilä, Reso (FIN)	1882	300	+	+	+
805	*Avena sativa*	Wisingsö	Visingsö (SWE)	1896	400	+	+	+*
806	*Avena sativa*	Ölandshavre	Stora Frö (SWE)	1896	300	+	+	+
921	*Avena sativa*	Local cultivar	Wuono (SWE)	1896	300	+	+	+
1131	*Avena sativa*	Gammal svensk	Kilanda (SWE)	1896	200	+	+	+
1604	*Avena sativa*	Local cultivar	Leksand (SWE)	1867	200	+	+	−
1839	*Avena sativa*	Fadrette	Stockholm (SWE)	1865	300	+	+	+
1881	*Avena sativa*	Sandy	Österäng (SWE)	1873	200	+	+	+
	Avena sativa	SW Belinda	Svalöv (SWE)	2006	>10,000	+	+	+
302	*Phleum pratense*	Local cultivar	Mattila (FIN)	1882	200	+	+	+
304	*Phleum pratense*	Local cultivar	Kankaanpä (FIN)	1882	200	+	+	+
308	*Phleum pratense*	Local cultivar	Haarois (FIN)	1882	200	+	+	+
1340	*Phleum pratense*	Local cultivar	Lagerlunda (SWE)	1896	200	+	+	+
1354	*Phleum pratense*	Local cultivar	Kolbäck (SWE)	1896	200	+	+	+*
1358	*Phleum pratense*	Local cultivar	Äs (SWE)	1896	200	+	+	+
1365	*Phleum pratense*	Local cultivar	Västerby (SWE)	1896	200	+	+	+
1735	*Phleum pratense*	Local cultivar	Illinois (US)	1876	200	+	+	+
1854	*Phleum pratense*	Weltruss	(BLR)	1873	200	+	+	+*
2644	*Phleum pratense*	Timotej	Unknown	1877	200	+	+	+

https://doi.org/10.1017/S0960258509990055 Published online by Cambridge University Press

Table 2. *Continued*

Acc. #	Species	Cultivar name	Growth location	Harvest year	DNA fragment size (bp)	PCR amplification 109 bp	350 bp	700 bp
	Phleum pratense	SW Ragnar	Svalöv (SWE)	2006	>10,000	+	+	+
323	*Pisum sativum*	Local cultivar	Hartsala (FIN)	1882	2000	+	+	+
329	*Pisum sativum*	Local cultivar	Kangais (FIN)	1882	2000	+	+	+*
1272	*Pisum sativum*	Local cultivar	Fogelsta (SWE)	1896	3000	+	+	+
1281	*Pisum sativum*	Lolländsk	Nyrup (SWE)	1896	500	−	+	−
1383	*Pisum sativum*	Local cultivar	Cederslund (SWE)	1896	500	−	+	−
1527	*Pisum sativum*	Stensärt	Hedvigsdal (SWE)	1918	500	+	+	+
1537	*Pisum sativum*	Fürst Bismark	Svalöv (SWE)	1918	6000	+	+	+
1553	*Pisum sativum*	Engelsk Sabel	Mjölby (SWE)	1918	3000	+	+	+
1618	*Pisum sativum*	Local cultivar	Ekholmen (SWE)	1868	500	+	+	+*
2335	*Pisum sativum*	Daniel O'Rourkes	Unknown	1877	4000	+	+	+
	Pisum sativum	SW Odalett	Svalöv (SWE)	2006	>10,000	+	+	+
1336	*Vicia sativa*	Småvicker	Borstad (SWE)	1896	400	− (+)	− (+)	− (−)
1337	*Vicia sativa*	Ölandsvicker	Eriksöre (SWE)	1896	1500	− (+)	− (+)	− (+)
1361	*Vicia sativa*	Fodervicker	Visingsö (SWE)	1896	300	+	+	+*
1398	*Vicia sativa*	Sötvicker	Idingstad (SWE)	1896	1000	−	−	+
1431	*Vicia sativa*	Sötvicker	Ekeby (SWE)	1896	4000	−	+	+*
1440	*Vicia sativa*	Sötvicker	Tistad (SWE)	1896	2000	−	+	+
1460	*Vicia sativa*	Storkärning vicker	Riddersvik (SWE)	1896	2000	−	+	−
1493	*Vicia sativa*	Förädlad sötvicker	Svalöv (SWE)	1918	2000	− (−)	− (+)	− (−)
1521	*Vicia sativa*	Förädlad gråvicker	Svalöv (SWE)	1918	2000	− (+)	− (+)	− (+)
1896	*Vicia sativa*	Vicker	Unknown	1864	2000	−	−	+
	Vicia sativa	SW fodervicker	Svalöv (SWE)	2006	>10,000	+	+	+
305	*Trifolium pratense*	Local cultivar	Mattila (FIN)	1882	1000	+	+	+
311	*Trifolium pratense*	Local cultivar	Sillanpää (FIN)	1882	1000	+	+	+
1343	*Trifolium pratense*	Local cultivar	Nibble (SWE)	1896	200	+	+	−
1344	*Trifolium pratense*	Local cultivar	Kullstad (SWE)	1896	200	−	−	−
1372	*Trifolium pratense*	Local cultivar	Västerås (SWE)	1896	300	+	+	+*
1377	*Trifolium pratense*	Local cultivar	Uttersberg (SWE)	1896	300	−	−	+
1380	*Trifolium pratense*	Local cultivar	Åkerstad (SWE)	1896	1000	+	+	+
2698	*Trifolium pratense*	Svensk	Unknown	1877	1000	+	+	+
2699	*Trifolium pratense*	Schlesisk	Unknown	1867	3000	+	+	+*
2700	*Trifolium pratense*	Steyermarker	Unknown	1877	1000	+	+	+
	Trifolium pratense	SW Fanny	Svalöv (SWE)	2006	>10,000	+	+	+
1494	*Beta vulgaris*	Barres halflång	Svalöv (SWE)	1918	1000	+	+	+
1495	*Beta vulgaris*	Golden Tankard	Landskrona (SWE)	1896	300	+	−	+
1507	*Beta vulgaris*	Röd Eckendorfer	Landskrona (SWE)	1896	300	+	+	+*
1520	*Beta vulgaris*	Tidiga sockerbetor	Svalöv (SWE)	1918	1000	+	+	+
1701	*Beta vulgaris*	Hvita sockerbetor	Illinois (US)	1876	3000	+	+	+
1707	*Beta vulgaris*	Egyptian	Illinois (US)	1876	300	+	+	+
1845	*Beta vulgaris*	Zvidlingsburger	Neuhof (GER)	1873	300	+	+	+
1846	*Beta vulgaris*	Kleinvands lebenen	Vitryssland	1873	3000	+	+	+
1848	*Beta vulgaris*	Bestehorn	Neuhof (GER)	1873	500	+	+	+*
1855	*Beta vulgaris*	Stora conciliatriee	Neuhof (GER)	1873	1000	+	+	+
	Beta vulgaris	Boltardy	Spekeröd (SWE)	2006	>10,000	+	+	+
1400	*Brassica napus*	Local cultivar	Smedby (SWE)	1896	200	+	+	+
1404	*Brassica napus*	Local cultivar	Wärnberg (SWE)	1896	1000	+	+	+
1512	*Brassica napus*	Local cultivar	Uppsala (SWE)	1896	1500	+	+	+*
1538	*Brassica napus*	Local cultivar	Örebro (SWE)	1896	1500	+	+	+
1697	*Brassica napus*	Winterrübsen	Lidingö (SWE)	1868	Nd	+	−	−
2468	*Brassica napus*	Uckermarker Jette	Unknown	1877	500	+	+	+
2470	*Brassica napus*	Quedlinburger	Unknown	1877	500	+	+	+
2490	*Brassica napus*	Rysk Kubja	Unknown	1877	1000	+	+	+
2924	*Brassica napus*	Purple top yellow Swedish	Unknown	1877	1000	+	+	+
2933	*Brassica napus*	Laing's Röda Gröntoppiga	Unknown	1877	500	+	+	+*
	Brassica napus	SW Carousel	Svalöv (SWE)	2006	>10,000	+	+	+

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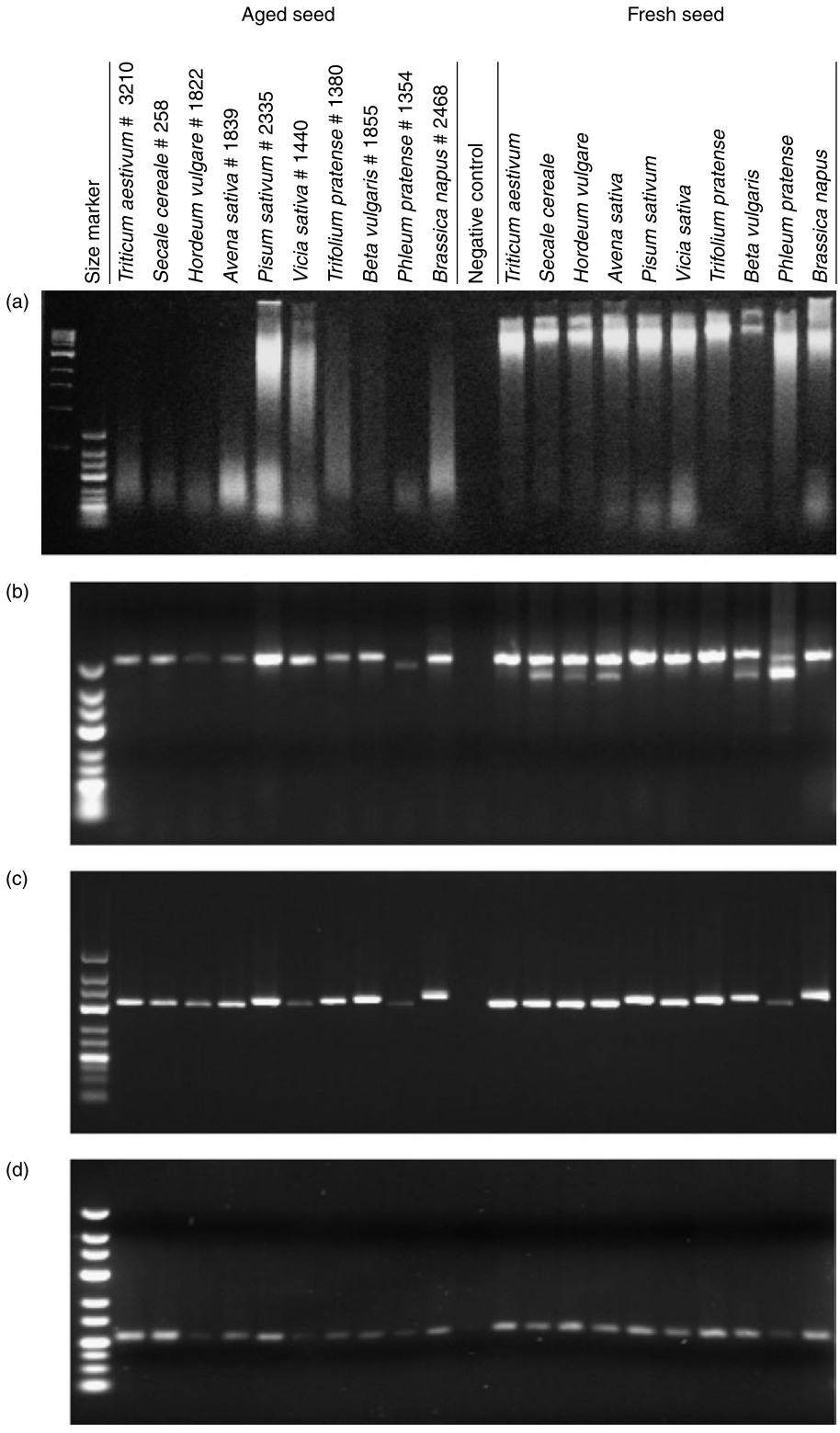

Figure 2. Examples of agarose gel electrophoresis of (a) extracted genomic DNA and (b–d) PCR amplification products from aged and fresh samples. Primer pairs are (b) ITS1 + ITS4 resulting in ~700 bp products; (c) ITS1 + ITS2 resulting in ~350 bp products; and (d) LR6 + LR17R resulting in 109 bp products. Agarose gels were stained with ethidium bromide and visualized by UV light.

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degrees of degradation (Table 1, Fig. 2a). In the grass seeds (cereals and *Phleum pratense*) the majority of DNA fragments were very short (230 ± 70 bp). DNA isolated from the *Fabaceae* species was less degraded, although some exceptions of considerably more severely degraded samples were found. The best preserved DNA was found in *Pisum sativum* (2200 ± 1850 bp) followed by *Vicia sativa* (1720 ± 1050 bp) and *Trifolium pratense* (900 ± 830 bp). In *Beta vulgaris* DNA yields were small and degree of degradation variable between accessions (1070 ± 1060 bp). Finally, *Brassica napus* showed an intermediate degree of degradation (860 ± 460 bp). DNA was not detected in two *Triticum aestivum* samples (#132 and #134), one *Secale cereale* sample (#77) and one *Brassica napus* sample (#1697).

The ability to characterize degraded DNA reliably was tested by PCR amplification of fragments using universal primers and subsequent sequencing to verify species, as described by Walters *et al.* (2006). Three different sets of primers were used, resulting in amplification products of ~700 bp, ~350 bp and 109 bp in length. DNA isolated from fresh seed samples readily amplified (Fig. 2b–d, right half of gels), and PCR products were frequently obtained from aged seeds (Fig. 2b–d, left half of gels), with 76, 86 and 84 samples of the 100 tested providing amplification products for the ~700, 350 and 109 bp fragments, respectively (Table 2). PCR products were not obtained in the highly degraded grain samples for which DNA was not detected (*Triticum aestivum* #132 and #134, *Secale cereale* #77). PCR products were obtained in *Trifolium pratense* #1344 and *Vicia sativa* #1336, #1337, #1493 and 1521 after DNA extracts were diluted 1:10. Negative extraction controls did not result in any amplification product.

Amplified ~700 bp products were sequenced from two of the aged samples from each species (indicated by * in Table 2), and species identification was confirmed by a BLAST search of GenBank (May 2008) for 19 of the 20 samples. The obtained sequence from the amplification of the *Phleum pratense* #1354 extract had 97% identity with *Aspergillus* sequences. The amplification of fungal DNA instead of *Phleum pratense* DNA was also visible as a fragment length polymorphism (Fig. 2b) resulting in a shorter fragment.

To test whether DNA polymorphisms in single-copy nuclear genes could be detected we analysed the 11 *Pisum sativum* and *Hordeum vulgare* samples (Table 2) with microsatellite markers (Table 3). DNA extracted from *Pisum sativum* and *Hordeum vulgare* had the longest and shortest average fragment sizes, respectively. Size of amplified microsatellite products were visualized on high-density agarose gels (Fig. 3) and quantified using capillary gel electrophoresis and confocal laser scanning.

For each species, six primers for fragments ranging in size from 156 to 300 bp gave polymorphic products for a total of 32 and 24 alleles in *Pisum sativum* and *Hordeum vulgare*, respectively (Table 3). All 11 samples within each species had some distinct alleles. In *Pisum sativum* all six markers could be amplified in all samples. Amplification was less successful in *Hordeum vulgare* samples. For example, only three of the six markers could be amplified in accession #737. For all other samples, all or nearly all markers amplified well and only the occasional marker (AF043094A) failed to amplify. The presence of non-amplifying null alleles at these loci cannot be ruled out. A high degree of homozygosity in both *Pisum sativum* and *Hordeum vulgare* is indicated by a single band for each

Table 3. Marker name and observed fragment sizes, chromosomal location, number of detected alleles and number of accessions with amplified product for the microsatellite markers tested in the *Pisum sativum* and *Hordeum vulgare* samples

Marker	Species	Chromosome location	Fragment size (bp)	Detected alleles	Accessions with amplified product
AA278[a]	*Pisum sativum*	3	170–180	4	11
AB71[a]	*Pisum sativum*	6	161–181	8	11
AB141[a]	*Pisum sativum*	3	158–216	6	11
AC58[a]	*Pisum sativum*	5	226–258	4	11
AD73[a]	*Pisum sativum*	3	248–296	5	11
AD83[a]	*Pisum sativum*	2	275–291	5	11
EBmac0827[b]	*Hordeum vulgare*	7	124–134	3	11
EBmag0705[b]	*Hordeum vulgare*	3	149–159	4	8
Bmag0613[b]	*Hordeum vulgare*	6	182–211	7	9
Bmag0378[b]	*Hordeum vulgare*	2	156–162	4	11
AF043094A[b]	*Hordeum vulgare*	5	166–168	2	2
Bmag0718[b]	*Hordeum vulgare*	1	172–182	4	9

[a] Markers from Loridon *et al.* (2005).
[b] Markers from Ramsay *et al.* (2000).

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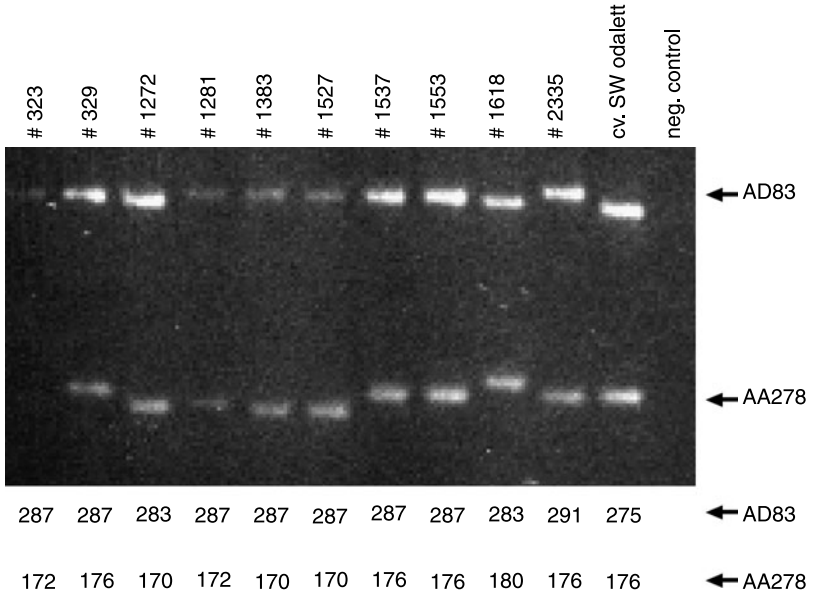

Figure 3. Examples of DNA polymorphisms detected by microsatellite markers. The figure shows markers AD83 and AA278 amplified from DNA extracted from ten *Pisum sativum* accessions and a modern pea cultivar. Amplification products were separated on a 3.5% MetaPhor agarose gel and stained with ethidium bromide for visualization. Fragment sizes (bp) determined by MegaBACE are indicated by the numerals below the gel.

microsatellite (Fig. 3 gives sample data for *Pisum sativum*) and is expected in inbreeding species. However, genotyping errors resulting from differential amplification of alleles at a locus (called allelic drop-out) are common in deteriorated DNA samples (Gagneux *et al.*, 1997) and require additional experiments before complete homozygosity can be concluded.

To validate results by biological relevance we estimated F_{ST} values between the two-row and six-row *Hordeum vulgare* samples. The two types of *Hordeum vulgare* are known to be well genetically segregated (Kolodinska Brantestam *et al.*, 2007). Average F_{ST} across the markers was 0.27, suggesting moderate to high genetic differentiation. The degree of differentiation differed between markers, with one allele not present in two-row accessions being fixed in six-row accessions for one marker, and another marker being close to monomorphic in the two-row accessions. Other markers showed little differentiation.

Discussion

Aged and ancient plant material has lately gained attention for its utility in analyses of plant evolutionary and breeding history with the aid of DNA techniques. As recently reviewed by Jones *et al.* (2008), in such studies herbaria and seed collections can serve as excellent complements to living and archaeological material. However, depending on the questions asked, the value of the materials will depend on several factors: (1) Can the origin and age of the material be correctly determined? (2) Is the quality of the seed DNA sufficient to allow for correct PCR amplification? (3) Is the amount of seed sufficient to be considered representative? This is especially important when the aim is to perform population genetic analyses. In this paper we have evaluated the hitherto unexplored KSLA seed collection for its research potential.

Most samples within the KSLA seed collection are dated with the year, in several cases the harvest day, and information on cultivar name and/or growing location is given on the majority of samples. Storage of seed in sealed glass containers protects them from decay and microbial infestation, as was evident in the KSLA collection where intact containers were free of infestation. The importance of container type for longevity of seeds in storage of viable seed has been stressed by Gómez-Campo (2006) and, most likely, the same features apply for storing non-viable seeds.

Detectable amounts of DNA could be extracted from single seeds of the cereal species, *Pisum sativum* and *Vicia sativa*, although for the other species bulked samples had to be used. The utility of bulked samples for genetic analysis is limited as equal contribution of single samples to the DNA pool cannot be assumed. However, bulk genotyping may provide insight into the presence of certain alleles in a population. In contrast, the species where genotyping of single individuals is possible are more interesting for population genetic studies, both for comparisons within and between accessions. Many accessions in

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the KSLA collection consist of hundreds or thousands of seeds, meaning that, at least for cereals, each sample is collected from a fairly high number of individual plants. Although the exact sampling procedures are unknown, it is reasonable to assume that the seeds are representative of the original population.

Large differences were observed in the quality of the DNA extracted from aged seeds (Table 2). Stability of DNA seems to be correlated roughly with seed longevity (Walters *et al.*, 2005), with longer fragments found in *Pisum sativum* and *Vicia sativa* accessions, intermediate stability in *Beta vulgaris*, *Brassica napus* and *Trifolium pratense* accessions, and most deterioration found in accessions of the grasses. Seed morphology may play a role here, with large, hard-shelled seeds better protecting DNA from degradation.

PCR amplification was usually successful when the size of the amplified product was less than the average size of the extracted DNA fragments. An exception is *Vicia sativa* with proportionally long DNA fragments preserved but amplification by PCR being problematic. Inhibitory substances were most likely present in *Vicia sativa* and PCR amplification was more successful after a reduction of the amount of template.

In seeds, as with all biological materials, there is a relationship between age and DNA quality (e.g. Walters *et al.*, 2006). However, within the short timespan tested here (90–145 years) we could not clearly correlate DNA detoriation with age. Several of the oldest samples (from 1865) permitted PCR amplification of the ~700 bp product and the only samples yielding DNA neither detectable on gels nor possible to amplify by PCR was *Triticum aestivum* #132 and #134 and *Secale cereale* #77. These samples all come from the same sub-collection – a donation from the French breeding company Vilmorin et Andrieux 1908. Why these samples have more severely damaged DNA than the others is unclear and further analyses are required to conclude whether this specific sub-collection has DNA of inferior quality.

We have shown that single-copy DNA such as microsatellite markers can be amplified readily in DNA from single seeds. The use of such markers is desirable for several reasons. The use of species-specific primers is preferable to general primers to avoid amplification of, for example, fungal DNA. Furthermore, microsatellite markers are commonly used for analyses of population genetics, historical isolation and genetic differentiation within a species (Selkoe and Toonen, 2007). Here we show that microsatellite markers can be used to detect DNA polymorphisms in individual seeds of both the relatively preserved DNA of *Pisum sativum* and the more degraded DNA of *Hordeum vulgare* from the 19th century.

Due to great availability in crop species, species specificity and low requirement for long DNA fragments, microsatellites have also been used in studies of ancient and aged plant material. For example, in 2600- to 1700-year-old *Vitis vinifera* seeds, Manen *et al.* (2003) could detect polymorphism with three primer pairs. From *Zea maize* seeds and cobs, 400–1300 years old, Lia *et al.* (2007) could amplify products with three different primer pairs, although no polymorphism was detected. Kobayashi *et al.* (2006) amplified 19 different microsatellite markers in *Oryza sativa* seeds that were almost 100 years old. However, such results have to be evaluated carefully, as microsatellites are prone to slippage during amplification, not the least when the template DNA is deteriorated (Gugerli *et al.*, 2005). One way to validate results is to evaluate them in the context of breeding history. In this study, we could distinguish clearly between the six-row and two-row barley accessions. While both the number of markers and individuals are too small to draw any major conclusions regarding population structure, it is nevertheless clear that the KSLA material will be useful for future population genetic studies.

In conclusion, we have shown that material from an extensive 19th-century seed collection permits DNA extraction and analysis. The well-documented material with high biological integrity opens up possibilities for exploring several issues concerning crop evolution, agricultural history in northern Europe and preservation of genetic resources. Genetic variation among landraces and obsolete cultivars can be studied, as well as genetic shifts through selection during the early decades of modern plant breeding. Comparisons between original populations of collected seed and those regenerated to maintain supply or viability may show the potential effects of genetic drift and contamination during gene-bank maintenance. Thus, DNA technology has increased the information value of unviable seeds stored in historical collections to levels unimagined by those who originally deposited the seeds in the museum.

Acknowledgements

We thank Annette Molbaek and Åsa Schippert at IBK, Linköping University for skilful assistance with MegaBACE. Curator Johan Åkerlund at the Swedish Museum of Cultural History is acknowledged for providing the historical documentation of the seed collection. This work was funded by the Lagersberg Foundation, the Swedish Board of Agriculture, the Royal Swedish Academy of Agriculture and Forestry, the Nilsson-Ehle foundation and Carl XVI Gustafs 50-year anniversary fund for science, technology and environment.

https://doi.org/10.1017/S0960258509990055 Published online by Cambridge University Press

References

Blatter, R.H.E., Jacomet, S. and Schlumbaum, A. (2002) Spelt-specific alleles in HMW glutenin genes from modern and historical European spelt (*Triticum spelta* L.). *Theoretical and Applied Genetics* **104**, 329–337.

Börner, A., Chebotar, S. and Korzun, V. (2000) Molecular characterization of the genetic integrity of wheat (*Triticum aestivum* L.) germplasm after long-term maintenance. *Theoretical and Applied Genetics* **100**, 494–497.

Chebotar, S., Röder, M.S., Korzun, V. and Börner, A. (2002) Genetic integrity of *ex situ* genebank collections. *Cellular and Molecular Biology Letters* **7**, 437–444.

Doebley, J.F., Gaut, B.S. and Smith, B.D. (2006) The molecular genetics of crop domestication. *Cell* **127**, 1309–1321.

FAO (1997) *The state of the world's plant genetic resources for food and agriculture*. Rome, FAO.

Freitas, F.O., Bendel, G., Allaby, R.G. and Brown, T.A. (2003) DNA from primitive maize landraces and archaeological remains: implications for the domestication of maize and its expansion into South America. *Journal of Archaeological Science* **30**, 901–908.

Gagneux, P., Boesch, C. and Woodruff, D.S. (1997) Microsatellite scoring errors associated with noninvasive genotyping based on nuclear DNA amplified from shed hair. *Molecular Ecology* **6**, 861–868.

Gómez-Campo, C. (2006) Erosion of genetic resources within seed genebanks: the role of seed containers. *Seed Science Research* **16**, 291–294.

Gugerli, F., Parducci, L. and Petit, R.J. (2005) Ancient plant DNA: review and prospects. *New Phytologist* **166**, 409–418.

ISTA (1999) International rules for seed testing. *Seed Science and Technology* **27** (**Supplement**), 1–333.

Jaenicke-Després, V., Buckler, E.S., Smith, B.D., Gilbert, M.T.P., Cooper, A., Doebley, J. and Pääbo, S. (2003) Early allelic selection in maize as revealed by ancient DNA. *Science* **302**, 1206–1208.

Johansson, P., Aarsrud, S. and Öberg, A.-C. (2003) Det underbara huset: Vänersborgs museum. *Vänersborgs museums skriftserie* **1**, 115–122.

Jones, H., Lister, D.L., Bower, M.A., Leigh, F.J., Smith, L.M.J. and Jones, M.K. (2008) Approaches and constraints of using existing landraces and extant plant material to understand agricultural spread in prehistory. *Plant Genetic Resources*, (in press). doi: 10.1017/S1479262108993138.

Kobayashi, A., Ebana, K., Fukuoka, S. and Nagamine, T. (2006) Microsatellite markers revealed the genetic diversity of an old Japanese rice landrace 'Echizen'. *Genetic Resources and Crop Evolution* **53**, 499–506.

Kolodinska Brantestam, A., von Bothmer, R., Dayteg, C., Rashal, I., Tuvesson, S. and Weibull, J. (2007) Genetic diversity changes and relationships in spring barley (*Hordeum vulgare* L.) germplasm of Nordic and Baltic areas as shown by SSR markers. *Genetic Resoures and Crop Evolution* **54**, 749–758.

Lia, V.V., Confalonieri, V.A., Ratto, N., Hernández, J.A.C., Alzogaray, A.M.M., Poggio, L. and Brown, T.A. (2007) Microsatellite typing of ancient maize: insights into the history of agriculture in southern South America. *Proceedings of the Royal Society B* **274**, 545–554.

Lister, D.L., Bower, M.A., Howe, C.J. and Jones, M.K. (2008) Extraction and amplification of nuclear DNA from herbarium specimens of emmer wheat: a method for assessing DNA preservation by maximum amplicon length recovery. *Taxon* **57**, 254–258.

Loridon, K., McPhee, K., Morin, J., Dubreuil, P., Pilet-Nayel, M.L., Aubert, G., Rameau, C., Baranger, A., Coyne, C., Lejeune-Hènaut, I. and Burstin, J. (2005) Microsatellite marker polymorphism and mapping in pea (*Pisum sativum* L.). *Theoretical and Applied Genetics* **111**, 1022–1031.

Manen, J.-F., Bouby, L., Dalnoki, O., Marinval, P., Turgay, M. and Schlumbaum, A. (2003) Microsatellites from achaeological *Vitis vinifera* seeds allow tentative assignment of the geographical origin of ancient cultivars. *Journal of Archeological Science* **30**, 721–729.

Morrison, L.A. (2001) The Percival Herbarium and wheat taxonomy: yesterday, today and tomorrow. pp. 65–80 *in* Caligari, P.D.S.; Brandham, P.E. (Eds) *Wheat taxonomy: the legacy of John Percival*. The Linnean Society of London Special Issue No. 3. London, Academic Press.

Poulsen, G., Holten, C. and von Bothmer, R. (2006) Identification and revival of low viability seed samples. *Genetic Resources and Crop Evolution* **53**, 675–678.

Poulsen, G., Holten, C. and von Bothmer, R. (2007) AFLP similarities among Danish cultivars of fodder beet (*Beta vulgaris* L. subsp. *vulgaris* var. *rapacea* Koch). *Genetic Resources and Crop Evolution* **54**, 1105–1115.

Ramsay, L., Macaulay, M., Degli Ivanissevich, S., MacLean, K., Cardle, L., Fuller, J., Edwards, K.J., Tuvesson, S., Morgante, M., Massari, A., Maestri, E., Marmiroli, N., Sjakste, T., Ganal, M., Powell, W. and Waugh, R. (2000) A simple sequence repeat-based linkage map of barley. *Genetics* **156**, 1997–2005.

Selkoe, K.A. and Toonen, R.J. (2007) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters* **9**, 615–629.

Steiner, A.M., Ruckenbauer, P. and Goecke, E. (1997) Maintenance in genebanks, a case study: contaminations observed in the Nürnberg oats of 1831. *Genetic Resources and Crop Evolution* **44**, 533–538.

Tanksley, S.D. and McCouch, S.R. (1997) Seed banks and molecular maps: unlocking genetic potential from the wild. *Science* **277**, 1063–1066.

Walters, C., Wheeler, L.M. and Grotenhuis, J.M. (2005) Longevity of seeds stored in a genebank: species characteristics. *Seed Science Research* **15**, 1–20.

Walters, C., Reilley, A.A., Reeves, P.A., Baszczak, J. and Richards, C.M. (2006) The utility of aged seeds in DNA banks. *Seed Science Research* **16**, 169–178.

White, T.J., Brims, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. pp. 315–322 *in* Innis, M.; Gelfand, D.; Sninsky, J.; White, T. (Eds) *PCR protocols: a guide to methods and applications*. San Diego, Academic Press.

Wright, S. (1951) The genetical structure of populations. *Annals of Eugenics* **15**, 323–354.

Received 9 July 2008
accepted after revision 20 March 2009
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https://doi.org/10.1017/S0960258509990055 Published online by Cambridge University Press