Seed Science Research (2006) **16**, 179–191 DOI: 10.1079/SSR2006251

Seed remains of common millet from the 4th (Mongolia) and 15th (Hungary) centuries: AFLP, SSR and mtDNA sequence recoveries

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Abstract

Seed remains of common millet (*Panicum miliaceum* L.) were excavated from sites of AD 4th-century Darhan (Mongolia), and AD 15th-century Budapest (Hungary). Because the 15th-century medieval grains looked so intact, a germination test was carried out under aseptic conditions, which resulted in swelling of the grains but no cell proliferation or germination. Ancient DNA (aDNA) was extracted from the aseptic grains; analysed for amplified fragment length polymorphisms (AFLP), simple sequence repeats (SSR) and mitochondrial DNA (mtDNA); and compared with the modern millet cultivar 'Topaz'. AFLP analysis revealed that extensive DNA degradation had occurred in the 4th-century ancient millet, resulting in only 2 (1.2%) AFLP fragments (98.8% degradation) amplified by *Mse*CAA–*Eco*AGT, compared to the 15th-century medieval millet, with 158 (40%) fragments (60% degradation), and modern millet cultivar 'Topaz' with 264 fragments (100%). *Eco*AGT–*Mse*-CAA was found to be the most effective selective-primer combination for the analysis of medieval and modern millet. Eight AFLP fragments were sequenced after re-amplification and cloning. Microsatellite (SSR) analysis at the nuclear *gln*4, *sh*1, *rps*28 and *rps*15 loci revealed one SNP (single nucleotide polymorphism) at the 29th position (A → G) of *rps*28 locus, compared to modern millet. An mtDNA fragment (*Mbo*I), amplified at the 18S–5S ribosomal DNA (rDNA) locus in the medieval millet, showed no molecular changes compared to modern millet. The results underline the significance of aDNA extraction and analysis of excavated seeds for comparative analysis and molecular reconstruction of ancient and extinct plant genotypes.

Keywords: ancient DNA, excavated seeds, *Panicum miliaceum*

Introduction

Common millet (*Panicum miliaceum*, $2n = 4x = 36$) is one of the most ancient grain crops, with the oldest historical reports from 5000–3200 BC (Ho, 1977). However, various plant remains from *c.* 12,000–8000 BP have been recovered from the Hoabinhian culture (Gorman, 1969; Walters, 1989). *Panicum* became a typical food of Sumer and northern India, together with barley (*Hordeum vulgare*), in about 2500 BC. For the nations of steppic Scythia, such as the Celts or Hungarians in 2000 BC, common millet was the first crop to produce two harvests in 1 year. In the ancient Chinese 'Book of Poetry' (*Shih Ching*), written about 1000–500 BC, nine poems mention common millet (Keng, 1974). This crop spread from the Steppes through Europe via tribes of the Celts, Huns, Avars and Hungarians, and also through the region of the 'Fertile Crescent' and Africa (Harlan, 1971). It was the *milium* (millet) of Romans (Smith, 1976). Millet was introduced to North America in the 17th century (Colosi and Schaal, 1997). New cultivars were registered recently.

Ancient DNA (aDNA) samples, recovered from excavated remains of plants and animals, supply unique materials not only for the analysis of post-mortem DNA degradation (Threadgold and Brown, 2003), but also for tracing crop domestication and microevolution (Brown, 1999), with a final aim for

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complete genome reconstruction of extinct organisms (Cooper *et al.*, 2001; Pääbo *et al.*, 2004) and genotypes (Szabo *et al.*, 2005). In this study, we present the aDNA analyses of 1600-year-old common millet excavated in Mongolia, and 600-year-old millet from a 15th-century site in Hungary, together with a comparison with the modern cultivar 'Topaz' as a control. Amplified fragment length polymorphism (AFLP) analysis was used to amplify aDNA fragments in high numbers and to estimate the degradation of aDNA. Locus-specific microsatellites (simple sequence repeats, SSR) were used to show authenticity of the *Panicum* analysed, and to amplify aDNA at the nuclear gene loci of *gln*4, *sh*1, *rps*28 and *rps*15. High copy number ancient mitochondrial DNA at the 18S–5S ribosomal DNA (rDNA) locus (*Mbo* I) was also recovered and analysed.

Materials and methods

Seed samples

Seed remains of common millet (*P. miliaceum*) from the 4th-century site (third grave, Darhan, Mongolia, excavated in 1969) (50 seeds) and 15th-century sites (150 seeds) (eighth well, Mansion Teleki, King's Palace, Budapest, Hungary) (Nyekhelyi, 2003) were used in this study. Wet-sieved sediment samples were processed by floatation, followed by seed sorting and identification in the laboratory (see Fig. 1). For comparative analyses the modern common millet cultivar 'Topaz' (ABI, Tapioszele, Hungary) was included.

The excavated seeds at the Budapest site (see Table 2) were examined under a light microscope (Wild M32, Leica, Hungary) and identified to species based upon seed morphology, using the Schermann Manual (1966). Seeds were also compared to the samples of a seed bank of the Middle European Seed and Fruit Collections (Gyulai, 2000).

Aseptic culture

Seed remains were washed with detergent (3 min) and rinsed three times with distilled water (3 min), followed by surface sterilization with ethanol (70% v/v) for 1 min and a commercial bleaching agent (8% NaOCl w/v) for 1 min; followed by three rinses with sterile distilled water, according to general tissue culture technique, and incubated for 3 months in aseptic tissue culture medium F6 (see Fig. 2; Gyulai *et al.*, 2003) to eliminate contamination before DNA extraction. Grains of the modern variety were also surface sterilized. Exogenously and endogenously

Table 1. Data of microsatellites (simple sequence repeats; SSRs) and mitochondrial DNA (mtDNA) loci with primer pairs applied

SSRs (NCBI number)		Primer pairs (5′–3′)	Expected product size (bp)	T_m (°C)	PCR cycles	Core sequences observed	References
1	*gln*4 (D14577)	agc aga acg gca agg gct act ttt ggc aca cca cga cga	260	60	40	$(TTGCG)_2$	Chin *et al.* (1996)
2	*sh*1 (AF544115)	atc gaa atg cag gcg atg gtt ctc atc gag atg ttc tac gcc ctg aag t	250	68	40	$(AAG)_6$	Chin *et al.* (1996)
3	*rps*28 (AW424565)	aga cga acc cac cat cat ctt tc cgc ttg gca tct cca tgt ata tct	162	66	40	$(TC)_8$	Chin *et al.* (1996)
4	*rps*15 (AW062092)	aag aag aaa gag aag aag cac ggg gga cag ctc gta tta taa cct gcg	146	68	40	$(CAG)_5$	Chin *et al.* (1996)
5	mtDNA (Z11512)	gtg ttg ctg aga cat gcg cc ata tgg cgc aag acg att cc	1177	60	40	–	Al-Janabi *et al.* (1994); Petit *et al.* (1998)

Gene symbols: *gln*4, glutamine tRNA synthetase-4; *sh*1, sucrose synthase (*shrunken*-1); *rps*28, ribosomal protein S28; *rps*15: ribosomal protein S15; mtDNA, 18S–5S rDNA.

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Figure 1. Clumps of excavated 15th-century seeds (King's Palace, Budapest, Hungary) after wet-sieving and floatation, with the most frequent species indicated.

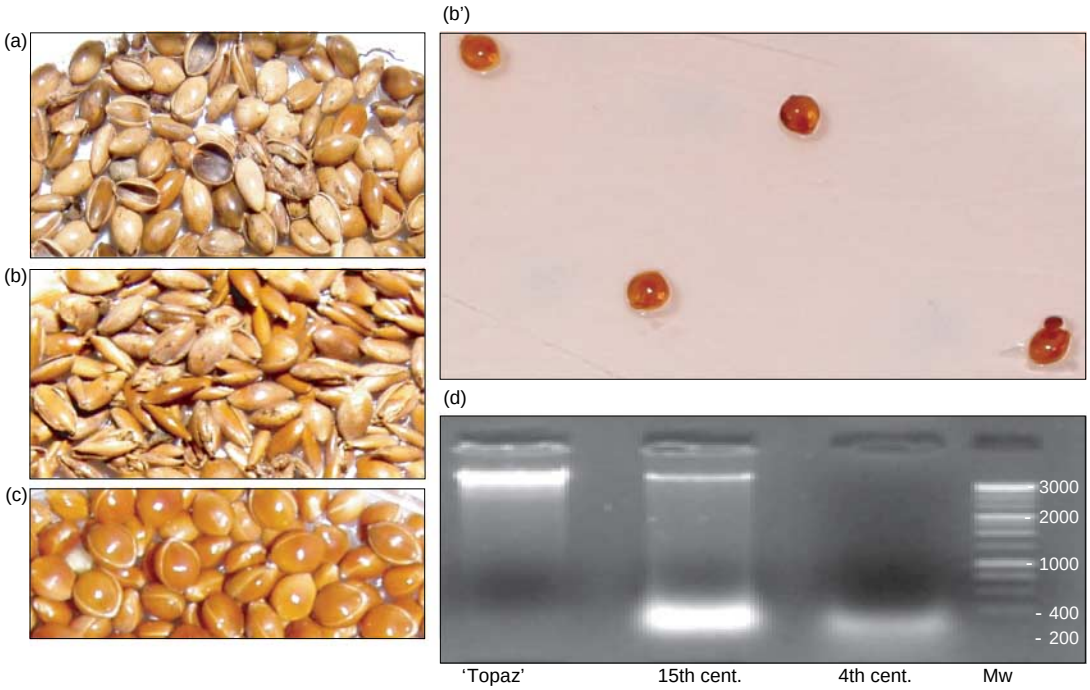

Figure 2. Surface sterilized grains (a)–(c), and DNA samples (d) (with molecular weight, Mw, markers in bp) of common millet (*Panicum miliaceum*) excavated from the 4th century (a) and 15th century (b), rehydrated and swelling on aseptic tissue culture medium (b′); and compared to modern millet cultivar 'Topaz' (c).

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Table 2. List and numbers (pieces) of identified seed remains (#) of excavated plant species (1–195) in the 15th-century site (King's Palace, Budapest, Hungary)

	Latin name	#		Latin name	#		Latin name	#
1	*Adonis aestivalis*	2	69	*Galium mollugo*	39	137	*Prunus fruticosus*	1025
2	*Aethusa cynapium*	2	70	*Galium spurium*	169*	138	*Prunus mahaleb*	425
3	*Agrostemma githago*	5516	71	*Glaucium corniculatum*	3	139	*Prunus padus*	7
4	*Ajuga chamaepitys*	335	72	*Glechoma hederacum*	1	140	*Prunus persica*	362
5	*Amaranthus lividus*	1243	73	*Heliotropium europaeum*	1	141	*P. spinosa (macrocarpa)*	35
6	*Anethum graveolens*	1622	74	*Hordeum murinum*	1		*P. spinosa (macrocarpa)*	50
7	*Anthemis tinctora*	2	75	*Hordeum vulgare*	8*		*P. spinosa* - a	194
8	*Apium graveolens*	12	76	*Humulus lupulus*	223		*P. spinosa* - b	216
9	*Arctium minus*	2	77	*Hyoscyamus niger*	4	142	*Punica granatum*	171
10	*Arctium tomentosum*	1	78	*Hypericum perforatum*	12	143	*Pyrus communis*	6682
11	*Avena sativa*	51*	79	*Juglans regia*	1609	144	*Pyrus* sp.	5290
12	*Ballota nigra*	1	80	*Lamium amplexicaule*	26	145	*Ranunculus repens*	117
13	*Brassica campestris*	21	81	*Lamium purpureum*	1	146	*Raphanus raphanistrum*	19
14	*Brassica oleracea*	34	82	*Laserpitium latifolium*	1	147	*Raphanus sativus*	52
15	*Bromus secalinus*	8*	83	*Lathyrus* sp.	3	148	*Reseda lutea*	1113
16	*Bryonia alba*	1	84	*Lens culinaris*	1*	149	*Rosa canina*	36
17	*Bupleurum rotundifolium*	1120	85	*Leontodon autumnalis*	1	150	*Rosa* sp.	584
18	*Calamintha acinos*	9	86	*Lepidium campestre*	3	151	*Rubus caesius*	24,579
19	*Camelina* sp.	97	87	*Linum austriacum*	3	152	*Rubus fruticosus*	2756
20	*Campanula* sp.	1	88	*Linum usitatissimum*	4	153	*Rubus idaeus*	1320
21	*Cannabis sativa*	1618	89	*Lithospermum offinale*	58	154	*Rumex acetosella*	8
22	*Carduus acanthoides*	4	90	*Lychnis flos-cuculi*	2	155	*Salvia nemorosa*	2
23	*Carex elata*	1	91	*Lycopus europaeus*	12	156	*Salvia verticillata*	104
24	*Carex flava*	2	92	*Malus domestica*	33,724	157	*Sambucus ebulus*	168
25	*Carex hirta*	32	93	*Malus silvestris*	21	158	*Sambucus nigra*	194
26	*Carex pallescens*	17	94	*Malus* sp.	8141*	159	*Saponaria officinalis*	14
27	*Carex silvatica*	1	95	*Malva alcea*	1	160	*Schoenoplectus lacustris*	14
28	*Carex tricarpellata*	3	96	*Malva neglecta*	1	161	*Sch. tabernaemontani*	2
29	*Carex vulpina/muricata*	24	97	*Marrubium peregrinum*	1307	162	*Schoenus nigricans*	2
30	*Castanea sativa*	352	98	*Marrubium vulgare*	3	163	*Scirpus maritimus*	1
31	*Caucalis platycarpos*	1	99	*Matricaria inodora*	12	164	*Secale cereale*	208*
32	*Centaurea cyanus*	6	100	*Melandrium noctiflorum*	4	165	*Setaria lutescens*	643
33	*Centaurea jacea*	560	101	*Mespilus germanica*	760	166	*Setaria viridis/verticillata*	37,001
34	*Centaurea scabiosa*	4	102	*Morus nigra*	39,670	167	*Silene alba*	151
35	*Cerealia*	1	103	*Muscari comosum*	2	168	*Silene dioica*	1
	Cerealia	39*	104	*Nepeta cataria*	3	169	*Silene vulgaris*	3
36	*Cerinthe minor*	16	105	*Neslea paniculata*	52	170	*Sinapis alba*	1
37	*Chenopodium album*	30,457	106	*Ocimum basilicum*	1	171	*Sinapis arvensis*	733
38	*Chenopodium ficifolium*	2	107	*Origanum vulgare*	2	172	*Solanum dulcamara*	57,962
39	*Chenopodium hybridum*	708	108	*Orlaya grandiflora*	33	173	*Solanum nigrum*	811
40	*Chrysanth. eucanthemum*	1	109	*Panicum miliaceum*	955,497	174	*Sonchus asper*	1
41	*Chrysanthemum segetum*	3		*Panicum miliaceum*	1442*	175	*Sonchus oleraceus*	20
42	*Cichorium intybus*	46	110	*Papaver dubium*	3	176	*Sorbus domestica*	1276
43	*Circaea lutetiana*	1	111	*Papaver rhoeas*	57	177	*Stachys annua*	847
44	*Citrullus lanatus*	54,415	112	*Papaver somniferum*	359,981	178	*Stachys arvensis*	43
45	*Conringia orientalis*	1	113	*Pastinaca sativa*	60	179	*Stellaria holostea*	1
46	*Convolvulus arvensis*	1	114	*Physalis alkakengi*	5517	180	*Stellaria media*	147
47	*Coriandrum sativum*	51	115	*Picris hieracioides*	5	181	*Taraxacum officinale*	2
48	*Cornus mas*	1936	116	*Piper nigrum*	1046	182	*Thalictrum flavum*	2
49	*Corylus avellana*	541	117	*Pisum sativum*	2*	183	*Thalictrum minus*	16
50	*Cucumis melo*	28,117	118	*Plantago major*	1	184	*Thlaspi arvense*	415
51	*Cucumis sativus*	11,783	119	*Poaceae*	1534	185	*Tilia* sp.	2
52	*Cuscuta europaea*	4	120	*Polygonum aviculare*	618	186	*Trifolium arvense*	1
53	*Cydonia oblonga*	351	121	*Polygonum mite/minus*	1	187	*Triticum aestivum*	221
54	*Cyperus fuscus*	1	122	*Polygonum persicaria*	49		*Triticum aestivum*	3*
55	*Cyperus longus*	1	123	*Potentilla erecta*	1	188	*Urtica dioica*	1
56	*Daucus carota*	71	124	*Potentilla reptans*	2	189	*Vaccaria pyramidale*	328
57	*Dianthus* sp.	3	125	*Primula elatior*	1	190	*Valerianella dentata*	51

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Table 2. *Continued*

	Latin name	#		Latin name	#		Latin name	#
58	*Diplotaxis muralis*	1	126	*Prunus amygdalus*	38	191	*Viburnum lantana*	10
59	*Echinochloa crus-galli*	4	127	*Prunus armeniaca*	6	192	*Viburnum opulus*	15
60	*Eleocharis palustris*	5	128	*Prunus avium*	9783	193	*Vicia hirsuta*	16
61	*Euphorbia cyparissias*	43	129	*Prunus cerasifera*	259	194	*Vitis vinifera*	241,231
62	*Euphorbia exigua*	1	130	*Prunus cerasus*	13,368	195	*Xanthium italicum*	1
63	*Euphorbia platyphyllos*	6	131	*Prunus domestica italica*	5			
64	*Fallopia convolvulus*	3275	132	*P. domestica institia*	14		Food remains (bread)	2*
65	*Ficus carica*	278,459	133	*P. domestica syriaca*	15		Food remains (gruel)	32*
66	*Fragaria vesca*	1,056,154	134	*P. domestica oeconomica*	651		Food remains (cooked)	12*
67	*Galeopsis ladanum*	1	135	*P. domestica Juliana*	736			
68	*Galium aparine*	60	136	*P. domestica oxicarpa*	111		Total	3,293,623

* Indicates carbonized samples.

contaminated grains infected by fungi and bacteria were eliminated from further analyses.

DNA extraction

Aseptic seed remains were ground in an aseptic mortar with liquid nitrogen in a laminar air-flow cabinet. aDNA was extracted by the CTAB (cetyltrimethylammonium bromide) method in batches, according to Yang (1997), Cooper and Poinar (2000) and Biss *et al.* (2003). Seed DNA of modern cultivars (0.1 g dry weight) was also extracted in CTAB buffer, followed by an RNase-A treatment (Sigma, St. Louis, Missouri, USA) for 30 min at 37°C. To avoid cross-contamination, ancient and modern samples were handled in separate laboratories using different laminar air-flow cabinets, pipettes, autoclaved tubes, mortars, pestles and thermocyclers, according to Szabo *et al.* (2005). The quality and quantity of extracted DNA were measured (2 µl) by a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Delaware, USA; BioScience, Budapest, Hungary). DNA samples were adjusted to a concentration of 30 ng/µl with double-distilled water (ddH_2O) and subjected to polymerase chain reaction (PCR) amplification according to Lagler *et al.* (2005).

PCR primers

Four nuclear simple sequence repeats (SSRs) at *gln*4, *sh*1, *rps*28 and *rps*15 loci and a ribosomal mtDNA at the 18S–5S rDNA locus were amplified (Table 1).

PCR

A minimum of two independent DNA preparations from each sample was used for PCR, following the basic protocols of amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995), SSR (Chin *et al.*, 1996) and mtDNA (Petit *et al.*, 1998). Each successful reaction with scorable bands was repeated at least twice. Negative controls including aDNA-free, primer-free, *Taq*-free and water were included in PCR runs.

SSR analysis

SSR fragments were separated (2 µl) by an automatic laser fluorometer (ALFexpress II DNA Analyser; Amersham Bioscience, Uppsala, Sweden; AP, Budapest, Hungary), according to Roder *et al.* (1998). Polyacrylamide gel electrophoresis (PAGE; 24% w/v) with a short thermoplate and 40-sample capacity, was run at 850 V, 50 mA, 50 W at 50°C for 120 min, prior to UV-linkage for 15 min. One primer of each primer pair was labelled with Cy5 fluorescent dye at the 5′-end (Sigma). For sequencing, SSR and mtDNA fragments (15 µl) were run and cut out from agarose (1.6% w/v) gels and purified in a spin column (Sigma). mtDNA fragments were digested with the restriction endonuclease *Mbo* I to facilitate sequencing, according to the manufacturer's protocol (Fermentas-Biocenter, Szeged, Hungary).

AFLP analysis

Undiluted aDNA samples (5.5 µl) were subjected to fAFLP (fluorescent AFLP) analysis, following the method of Vos *et al.* (1995) with modifications (Cresswell *et al.*, 2001; Skøt *et al.*, 2002). For digestion–ligation reactions, pairs of *Eco* RI–*Mse* I restriction endonucleases (REases) were used. The sequences of the preselective primer pairs were: *Eco-A* (gac tgc gta cca att c-a) and *Mse*-C (gat gag tcc tga gta a-c). For selective amplification, 24 primer combinations were used with 'JOE' (green) fluorescent-labelled **Eco*-primers. In primer combinations 1–12, the primer

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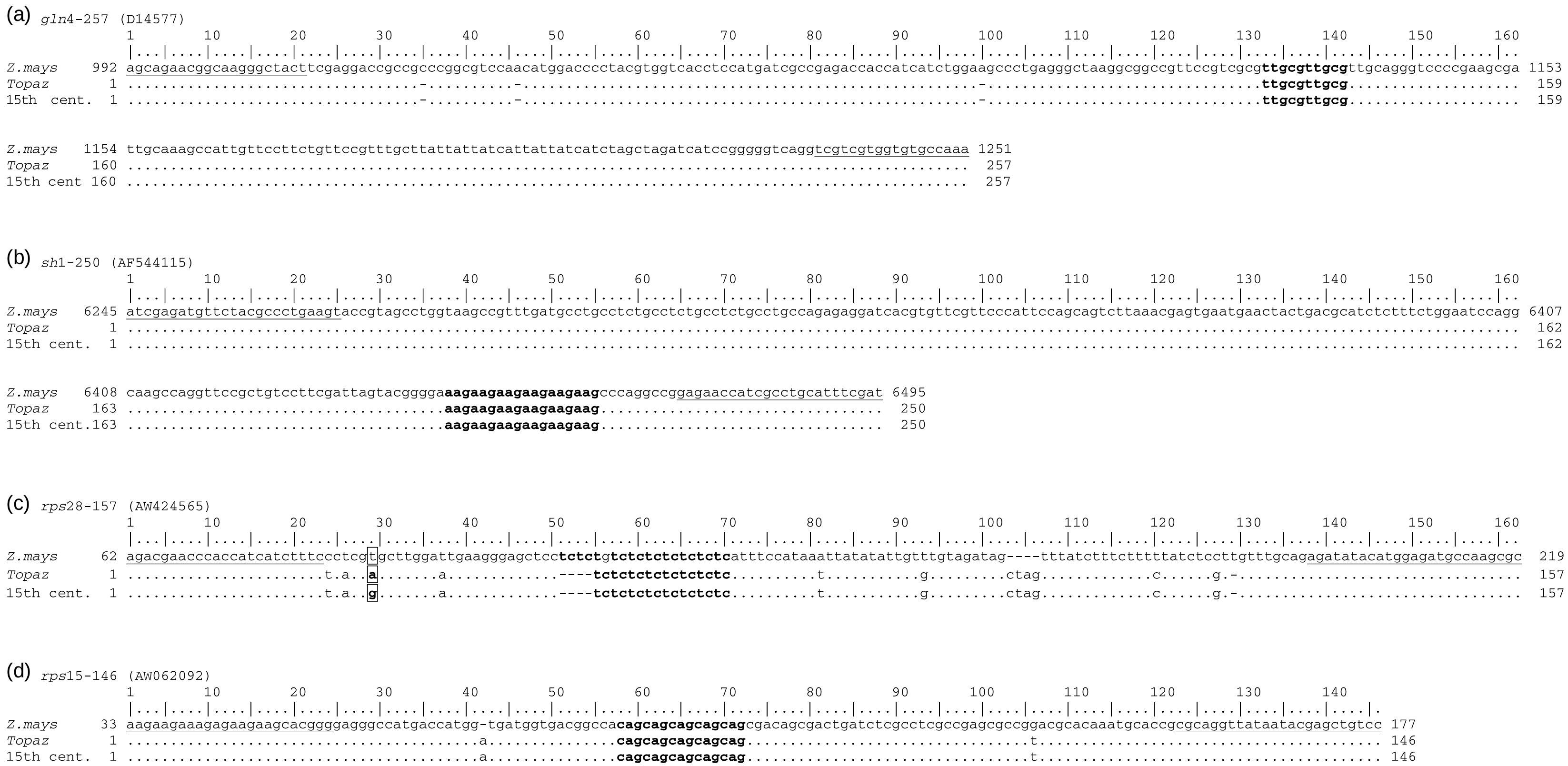

Figure 3. Consensus sequence alignments of nuclear microsatellite alleles at four simple sequence repeat (SSR) loci (a)–(d) of medieval millet (15th century) compared to modern millet cultivar 'Topaz' and maize with one single nucleotide polymorphism (SNP) at the 29th position (A → G) of the *rps*28 locus. Core nucleotides of SSRs (bold) and primer sequences (underlined) are indicated.

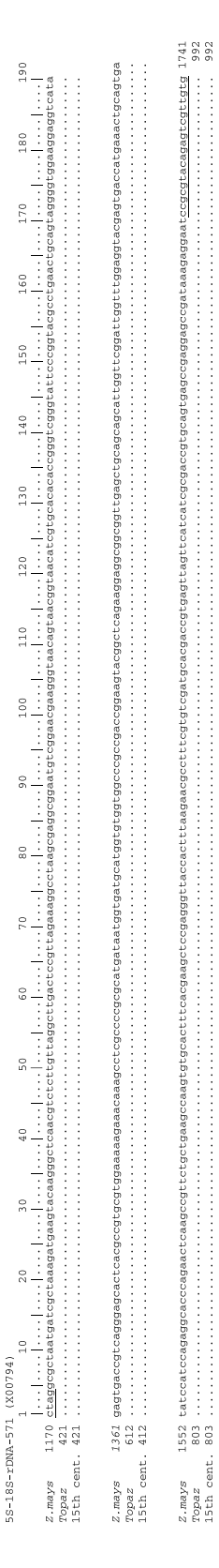

Figure 4. Consensus sequence alignments of *Mbo*I-571 fragments of ribosomal mtDNA at 5S–18S rDNA locus in the 15th-century millet compared to modern millet cultivar 'Topaz' and the NCBI database (X00794). The *Mbo*I restriction site (ctag) and the primer sequence (underlined) are indicated.

*Mse*CAC was combined with labelled primers of **Eco* -aaa, -aac, -aag, -aat, -aca, -acc, -agg, -act, -aga, -agc, -agg, -agt. In primer combinations 13–24, the labelled primer **Eco*AGT was combined with primers of *Mse* -caa, -cag, -cat, -cca, -ccc, -ccg, -cct, -cga, -cgc, -cgg, -cgt, -cta according to Gyulai *et al.* (2005). All oligonucleotides were supplied by Sigma Genosys (St. Louis, Missouri, USA), and enzymes were obtained from Roche Diagnostics (Basel, Switzerland). PCR-amplified AFLP fragments were subsequently denatured at 98°C for 5 min, and directly forwarded to an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA), using a G5 filter set in two repetitions, and analysed by ABI PRISM Genotyper 3.7 NT software at the range of 150–600 bp.

Fragment recovery

AFLP samples were loaded (8 μl) on to a 4.5% (w/v) PAGE sequencing gel (Sequi-Gen GT, Biorad, Hercules, California, USA) and run for 1.5 h at 100 W (2090 V, 48 mA), followed by silver staining (Promega, St. Louis, Missouri, USA). AFLP fragments recovered from gels were either reamplified (using 1 μl in a PCR reaction with non-labelled primer pairs of *Eco*AGT–*Mse*CAC and *Eco*AGT–*Mse*CAA), or cloned into pGEM-T Easy Vector System II, and transformed into a Jm109 competent cell (Promega). Inserts were cut out by *Eco* RI.

Sequencing

Fragments were subjected to automated fluorescent DNA sequencing (ABI PRISM 3100 Genetic Analyzer) and sequenced from both directions. Sequences were analysed by the computer program ChromasPro version 1.11 (Technelysium Pty Ltd, Tewantin, Queensland, Australia). Sequence alignments were analysed by BioEdit Sequence Alignment Editor (North Carolina State University, Raleigh, North Carolina, USA) and also by GCG-10 (Genetics Computer Group, Oxford Molecular Group Inc., Madison, Wisconsin, USA; Wisconsin Package, Version 10.3) software programs. BLAST (Basic Local Alignment Search Tool) analysis was carried out using the computer program from NCBI (National Center for Biotechnology Information, Bethesda, Maryland, USA).

Results

Excavation sites from the 4th century (Mongolia) and 15th century (Hungary) contained a great number of plant remains, including grains of common millet (*P. miliaceum*). The medieval site (Hungary) contained about 3 million plant remains of 195 species (Table 2),

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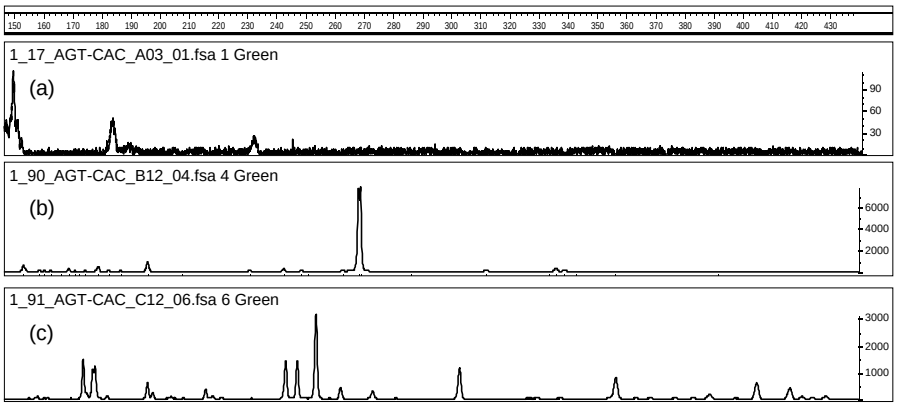

Figure 5. Samples of amplified fragment length polymorphism (AFLP) (*Eco*AGT–*Mse*CAC) fragment analysis (150–430 bp, with relative intensities of 30–6000) in the 4th-century (a) and 15th-century common millet (*Panicum miliaceum*) (b), compared to the modern millet cultivar 'Topaz' (c).

including 955,497 grains of common millet (Fig. 1). Intact grains from both sites were separated from damaged grains under a microscope. The 4th-century grains showed greater damage (Fig. 2). The ancient grains showed shrunken forms (Fig. 2) with a major loss of kernel; nevertheless, intact grains selected from the medieval sample showed swelling upon rehydration on tissue culture media after surface sterilization and incubation (Fig. 2). Aseptic seeds were separated and incubated individually (Fig. 2). Because the single grains had a low quantity of aDNA, 16 intact grains from 50 seed remains of 4th-century millet were pooled, and 78 intact grains from 150 seed remains of 15th-century millet were pooled to extract DNA, according to Michelmore *et al.* (1991).

SSR alleles at four loci – *gln*4 (257 nt), *sh*1 (250 nt), *rps*28 (157 nt) and *rps*15 (146 nt) – were amplified in the 15th-century millet and the modern millet cultivar 'Topaz'. No SSR amplification was observed in the 4th-century sample. The medieval SSR fragments showed identical alignments with modern millet and maize sequences, with only one SNP (single nucleotide polymorphism) at the 29th position (A → G) of the *rps*28 locus (Fig. 3).

When SSR sequences of medieval and modern millet were compared to maize (*Zea mays*) (NCBI database), three indels (insertion and deletions) and SNPs were observed in the *gln*4 allele of millets. There was consensus between the millets and maize at the *sh*1 locus. In the *rps*28 locus of millets, a $(ct)_2$ dinucleotide repeat deletion of the core sequence of SSR and several SNPs were observed compared to maize (Fig. 3). In the *rps*15 locus an insertion of A at the 42nd position, and a G → T nucleotide substitution (transversion) at the 106th position, were detected in the millets compared to maize (Fig. 3).

Of the organelle-specific primer pairs applied [chloroplast DNA (cpDNA): *trn* H, *trn* K, *rpo*C2 and *psb*C; and mtDNA: *nad*1B, *nad*1C, *cox* II and *cob*] from the collection of Petit *et al.* (1998) (data not shown), one of the 18S-5S ribosomal mtDNA primer pairs (Al-Janabi *et al.*, 1994) recognized a template in the medieval and modern millets. Sequence analysis of the restricted fragment *Mbo* I (571 bp) did not detect any nucleotide changes between the medieval and modern common millet (Fig. 4). No organelle aDNA was amplified in the 4th-century sample.

Table 3. Total numbers, %, and degradation (degr. %) of the fluorescent amplified fragment length polymorphism (fAFLP) fragments of common millets (4th and 15th centuries), compared to modern millet cultivar 'Topaz'. The selective AFLP primer combinations are: *Mse*CAC combined with (a) *Eco*AAT*, (b) *Eco*ACC*, and (c) *Eco*AGT*; and *Eco*AGT* combined with (d) *Mse*CAA, (e) *Mse*CAG, (f) *Mse*CAT, (g) *Mse*CCC, (h) *Mse*CCT, (i) *Mse*CGA, (j) *Mse*CGC and (k) *Mse*CTA

	fAFLP fragment number/selective primer pairs (a) to (k)											Total		
	(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)	(k)	No.	%	degr.%
4th century	–	–	2	–	–	–	–	–	–	–	–	2	0.8	99.2
15th century	10	18	24	34	29	12	16	5	5	3	2	158	60.0	40.0
'Topaz'	32	23	38	42	34	33	18	17	7	4	16	264	100.0	0

*Indicates a fluorescent-labelled primer.

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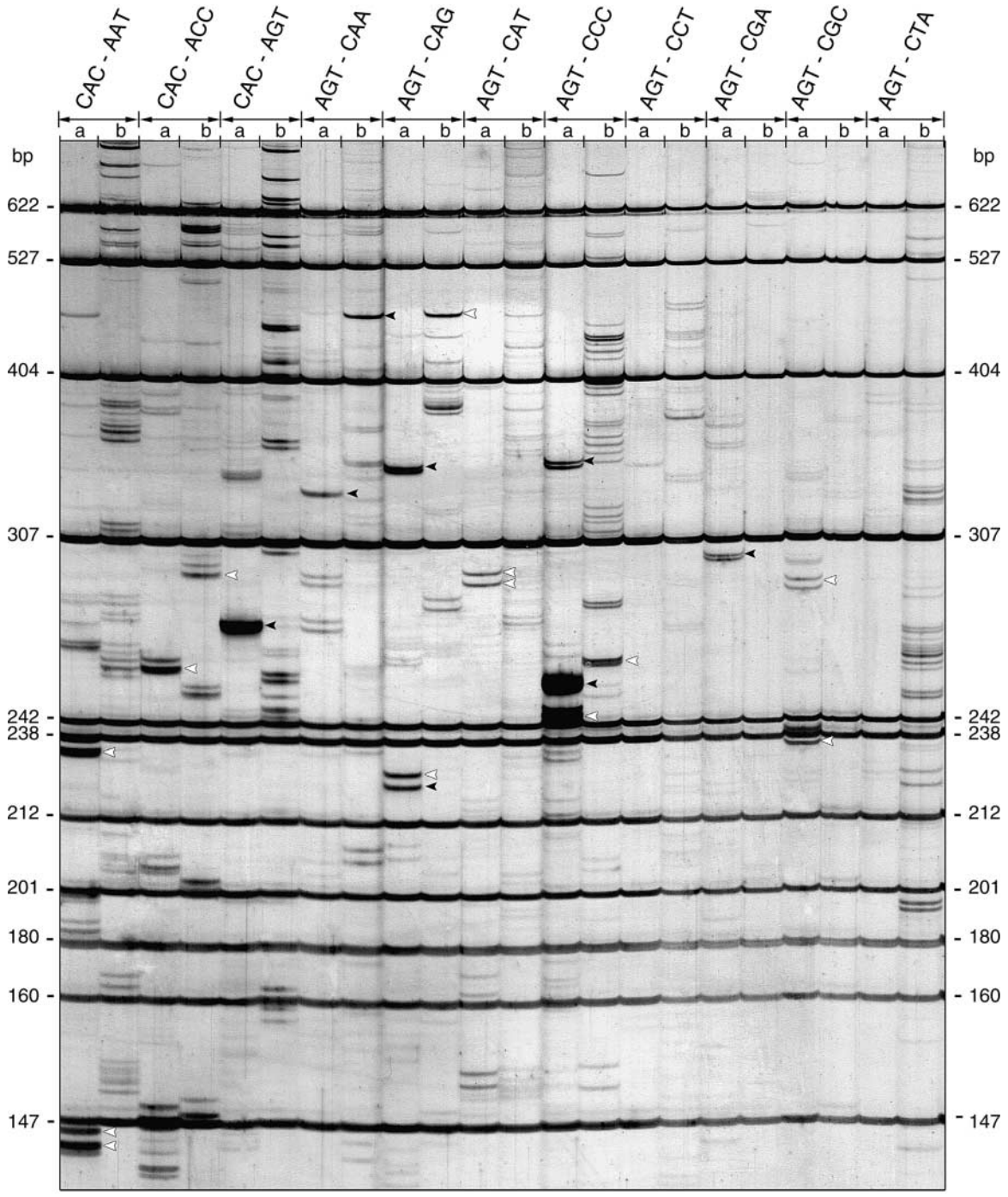

Figure 6. Polyacrylamide gel electrophoresis (PAGE) analysis of amplified fragment length polymorphism (AFLP) fragments of common millet (*Panicum miliaceum*) excavated from the 15th-century site (a) compared to the modern cultivar 'Topaz' (b). DNA molecular weight markers (622 to 147 bp), fragments recovered (white arrowheads) and sequenced (black arrowheads) are indicated.

AFLP analysis

Eleven of the 24 selective primer pairs produced sharp AFLP patterns in the samples (Fig. 5). The 4th-century sample revealed only two fragments (85 bp and 230 bp), both amplified by the *Eco*AGT–*Mse*CAC primer pair; 158 AFLP bands were amplified in the 15th-century sample compared to the 264 AFLP bands

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(1) *Eco*AGT-*Mse*CAG-225 – 15th cent.
gactgcgtaccaattc**agt**gtgaatgacggggatgaccggatcaaatcgattagaggtatggaagggattgtaaggcagatcagaaggctgtcg
ccgaagacggatatttgcttcgtctatacggcagccgacaagaatctggccgatccgttaccgttcaatatagccgttcatgaagaagtagctg
cccattacgctattcccg**ctg**ttactcaggactcatc

(2) *Eco*AGT-*Mse*CCC-252 – 15th cent.
gactgcgtaccaattc**agt**atccggataccaactatccctctcgtaatggttagattccatttggggtcgctgagtcatttcctgatacaagaa
cagaccaggcacgatcatgtacggggaggagctgcgtttgttctcaaatgccgctgctcctcttccgcggagcgcctctgtacccaaatgctgt
aggcggccatccccgatacacattccatatcgataaaccaaggag**ggg**ttactcaggactcatc

(3) *Eco*AGT-*Mse*CAC-272 – 15th cent. - (BAB04200)
gactgcgtaccaattc**agt**aggcctacctctttgacgacgagatacgttggaatcaagccaccgctgaaaaacatcgtaataacgaacgtcagc
atcaagacacgcccgtagggcagtacttttttggataacgcataagcagccaatatcgtcaacgccatactgaccaatgtgccgacaaccgtaa
taaacacggaattggtaaaggagtaaaagaactgcttgttcgtaagcacatactcaaacgacatc***gtg***ttactcaggactcatc

(4) *Eco*AGT-*Mse*CGA-298 – 15th cent.
gactgcgtacccaattc**agt**agaaagacagccaagacaagtccttcaggcatattctgtgccccgatggatatcgcgaccataggacctaaagc
ttcattgctactcgcataactaaatcctgtgctgagtccctccgggatgttatggataaatagagctataataaccaataaagatttggcatca
aaggatttgataccagaatcattctccacatctacatgaggaatattcttctcaataatatccagaacaacaataccaatcacaaaacctat**cg**
ttactcaggactcatc

(5a) *Eco*AGT-*Mse*CAA-331 – 15th cent.
gactgcgtacccaattc**agt**caaaaatcaacgtgcagttgaagagcgcgtgcgcgaagcactgcaaatggatcgcgcccgcgtgcaagttggcc
gtatttcacgctttggcttgctggagatgtctcgccagcgtctgcgcccttcattgaacgaaacctcaggtcacgtctgcccgcgctgcaacgg
ccaaggcaccattcgcggcacccgctcaatcgcgctggccatcttgcgtatgctggaagaagaagcgcagaaagagcgcagctcagaagtgcgc
gccctgacgccagtcgccgtcgcaagcttc**ttg**ttactcaggactcatc

(5b) *Eco*AGT-*Mse*CAA-462 – cv. Topaz - (AF050455)
gactgcgtaccaattc**agt**cgcgctttggaggacaaggcggaggatatccgcagagtcaacagaatcaacgaactccacagcagcctcagcgct
tcaaccagcagactcagcgcaactccaacccgcaaccgtaccgttcaggctatgccacgggtgcaccagtgcggaccaacaatccagtcacacc
ggtccaacccaacggctgtttcaaatgtggagaactggggcattatgctaacaactgcccgaagcgtaacrtgcagactcctcagaatcagagg
aacagtggacagaggcagactcctcagcagcagcagccgcgcaacagcaaccagacaccactgggcaacagaggtcaacagaactacgtgcgcg
gaaagggtgaatcatgtggctgcagaaacagctgaagaggctcccgatgttgtgctcggtacgttcc**ttg**ttactcaggactcatc

(6) *Eco*AGT-*Mse*CAG-344 – 15th cent.
gactgcgtacccaattc**agt**acaacgaatttctctcctctattcctggcatcagccagctcactcttctggtcatttgaaggcgtaacgatgat
gtattcatccagattaggaggtatggattgcagatcagccaggtccaaaaaccggatggaaacaccacgttgatgtgcttcatccctcatgccg
tccatcatctccgaaagggtagcatatacactgcgatgtggattgcataaccagacattcaacgtcgctgttgacgggttctcgtgctctttag
caatttcccggttggacacatacgttcctttacctttctttcg**ctg**ttactcaggactcatc

(7) *Eco*AGT-*Mse*CCC-345 – 15th cent.
gactgcgtacccaattc**agt**gtgacgctgtggggcaagaacctcaccaacaagcactacgcaagccgcgcggtcgattttggacaactgggatt
ttcgaccgtcagtttcggtgatcccagaaccttcggcctcacgctggacgctcaattctaggaacgaatgccatttggtcgccgcatcaagttt
atgcggcgaccagggctcatacttgttgggtgtgcaaagctaatcatgcggaaattgccagaaatctgccatttacaaacacacgagacacacg
ctcaaaaagcgctctcgctttcagttctttcactttgaatgtcg**ggg**ttactcaggactcatc

Figure 7. Sequence data of amplified fragment length polymorphism (AFLP) fragments of 15th-century common millet (*Panicum miliaceum*) (1)–(7), compared to the modern millet cultivar 'Topaz' (5b). Primer sequences of selective primer pairs of *Eco*A-gactgcgtaccaattc-***axx*** and the complementary sequence of *Mse*C-gatgagtcctgagtaa-***cxx*** are underlined.

detected in the modern common millet cultivar 'Topaz'. The average number of AFLP fragments per selective primer pair ranged from 0.2 (4th-century millet) and 14.4 (15th-century millet) to 24 ('Topaz'). The selective primer pair *Eco*AGT–*Mse*CAA was the most effective combination, generating 34 and 42 AFLP fragments in the 15th-century millet and 'Topaz', respectively (Table 3).

Twenty-one AFLP fragments were recovered from a PAGE gel (Fig. 6), and eight of them revealed identical sequences after either reamplification or cloning (Fig. 7). BLAST analysis revealed that one fragment (*Eco*AGT–*Mse*CAC-272) from the 15th-century millet showed significant similarity with the universal *Ugpe*, an ABC-type transporter (permease) gene (NCBI, BAB04200) (Fig. 7). A fragment (*Eco*AGT–*Mse*CAA-462) from the modern millet cultivar 'Topaz' showed significant similarity with the gypsy/Ty3-type retrotransposon (NCBI, AF050455). AFLP fragments amplified by the same

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primer pair (*Eco*AGT–*Mse*CAA) in the medieval and modern millet (5a and 5b in Fig. 7) did not show sequence homology, when amplified from different loci of their genomes. The two AFLP fragments from the 4th-century millet had such low intensities that further fragment purification was not successful.

Discussion

Common millet grains of the 15th-century site used in the present study appeared to be extremely well preserved due to anaerobic conditions in the slime of a deep well covered by water, apparently used as dust-hole in the Middle Ages (Nyekhelyi, 2003). These seeds looked intact, but were incapable of germinating: the half-life longevity of *P. miliaceum* is reported to be *c.* 12 years (Priestley, 1986). Nevertheless 15th-century grains showed swelling on tissue culture medium (Fig. 2) by water uptake, a primary event of seed germination (Bewley, 1997). Despite no germination, aseptic archaeo seeds, free of foreign DNA contamination, were obtained for further aDNA extraction and molecular analysis.

Agarose gel electrophoresis of the extracted total aDNA showed different degrees of degradation in the 4th- and 15th-century samples, compared to modern common millet (Fig. 2), probably due to hydrolytic and oxidative damage (Yang, 1997; Poinar *et al.*, 2003; Pääbo *et al.*, 2004). The quantities of aDNA extracted from two batches of 4th-century grains (0.083 g and 0.079 g produced 2.26 ng/μl and 1.97 ng/μl DNA, respectively) and those of 15th-century grains (0.089 g and 0.090 g produced 8.82 ng/μl and 6.71 ng/μl DNA, respectively) were much lower than the DNA samples from the modern common millet cultivar 'Topaz' (0.315 g and 0.371 g produced 417.3 ng/μl and 536.4 ng/μl DNA, respectively). The 15th-century aDNA showed less degradation than in 4th-century samples, with the presence of high molecular weight fragments (Fig. 2), probably not only because of the younger age of the samples, but also because of the cold, humid and anaerobic conditions in the medieval well. Successful aDNA extractions have been made from 400,000- to 10,000-year-old permafrost plant samples from Siberia (Willerslev *et al.*, 2003). The extensive DNA degradation in the 4th-century millet aDNA was probably due to the arid continental climatic conditions at the surface excavations of the sandy soil site in Mongolia.

In theory, microsatellites, as highly species-specific probes (Toth *et al.*, 2000), are optimal for aDNA analysis by excluding cross-reactions with contaminating microorganisms, or any ancient organisms and laboratory DNAs (Gugerli *et al.*, 2005). SSR analysis has been applied to *c.* 100-year-old herbarium samples of common reed (*Phragmites australis*) to track plant invasion in North America (Saltonstall, 2003). Melon (*Cucumis melo*)-specific SSRs were used to identify an *inodorus* type melon recovered from the 15th century (Szabo *et al.*, 2005). Allelic diversity of microsatellites was also reliably detected in aDNAs of 4000-year-old seagrass (*Posidonia oceanica*) (Raniello and Procaccini, 2002). However, the very fragmented aDNA (Fig. 2) of the 4th-century millet prevented SSR amplification in our study.

Unlike wheat, maize and *Lolium*, no database is available for common millet at present. Therefore, we selected maize-specific nuclear SSR markers for DNA-fishing in the aDNA microsatellites. At the *rps*28 locus, only one nucleotide change (A → G) was observed between 15th-century millet and modern millet cultivar 'Topaz' at the 29th position (Fig. 3), which might be the reason for the post-mortem hydrolytic deamination of adenine (A) to hypoxanthine, which pairs with cytosine (C) and can be read as guanine (G) in the PCR amplification (Threadgold and Brown, 2003). Because the A → G transition is located in the middle of the SSR sequence, it does not seem to be an artefact caused by *Taq* polymerase, which adds an A to the amplified fragment at the 3′ end of the sequence (Hofreiter *et al.*, 2001; Poinar *et al.*, 2003).

The theoretical opposite nucleotide transition from G to A in modern millet compared to medieval millet, as the result of microevolution, seems to be unlikely, since the relatively constant rate of mutation in evolution is longer than 600 years, even in crops that have been under accelerated evolution by selection pressure (Bromham and Penny, 2003). To compare, the mutation rate of human SSRs is about 10^{-3} to 10^{-5} per gamete (Bowcock *et al.*, 1994). Nevertheless, nuclear SSRs of aDNA of medieval melon (*Cucumis melo*), excavated from the same site as the millet in the present study, showed a high number of indels (13) in a 1383-bp-long sequence of eight microsatellite loci (Szabo *et al.*, 2005). Our results might indicate a more stable monocotyledonous millet genome compared to dicotyledonous melon, or more accelerated post-mortem aDNA degradation in the high-protein-containing melon seeds than in the high-carbohydrate-containing millet grains (Poinar and Stankiewicz, 1999).

Organelle mtDNA at the 5S–18S rDNA locus was amplified in the medieval sample, but not in the 4th-century sample. The *Mbo* I-571 fragment of the medieval sample revealed consensus sequence alignments with the modern cultivar 'Topaz' (Fig. 4), without SNPs, as expected. Multiple copy aDNA fragments – such as the highly conserved mtDNA, cpDNA and rDNA – were also reported to be highly amplified compared to double-copy (2*n*) nuclear aDNA sequences (Deguilloux *et al.*, 2002; Gugerli *et al.*, 2005). The reason for the unsuccessful reactions in the rest of the organelle-specific markers, including cpDNA (*trn* H, *trn* K, *rpo*C2

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and *psb*C) and mtDNA (*nad*1B, *nad*1C, *cox* II and *cob*) (Petit *et al.*, 1998), might be due to the expected long template fragments, which suggests a need for different protocols, such as long-PCR (Cheng *et al.*, 1994; Cooper *et al.*, 2001). Restorase DNA polymerase and GenomePlex WGA amplification (Sigma) have been used for amplification of DNA samples with limited quantities and qualities (Sun *et al.*, 2005).

In the AFLP analysis, only one of the seven ALF fragments (Fig. 6) showed identical plant DNA sequences in the medieval sample. Nevertheless, only AFLP (Table 3) was successful in the fragment recovery from the 4th-century millet, compared to SSR or mtDNA. This result underlines the useful application of AFLP with high amplification capacity in archaeogenetics (Allaby and Brown, 2003).

To conclude, the incubation of archaeobotanical samples of common millet under aseptic conditions provided contamination-free seed samples for aDNA isolation. The aDNA degradation evaluated by AFLP was extensive in the 1600-year-old (4th-century) sample (98.8%). In the 15th-century sample, with 40% AFLP degradation, a total of 2529-bp-long AFLP sequences was recovered. A further 1802-bp-long sequence was recovered from the 15th-century millet at five nuclear SSR and mtDNA loci; these sequences are the first records in a *Panicum* database. Since only one SNP was observed in the 15th-century millet, at the *rps*28 SSR locus, this indicates a genetically stable *Panicum* genome with good preservation conditions at the medieval excavation site.

Acknowledgements

The authors wish to thank Dr L. Skøt, S. Heywood and K. Skøt for contributing in the AFLP analysis. The project was supported by Hungarian Scientific Research Group OTKA-M-04 563, Organization for Economic Co-operation and Development grant OECD-JA00018642, the USA–Hungarian Fulbright Commission, and a Széchenyi Fellowship (SzÖ-4/2003).

References

Al-Janabi, S.M., McClelland, M., Petersen, C. and Sobral, B.W.S. (1994) Phylogenetic analysis of organellar DNA sequences in the Andropogoneae: Saccharinae. *Theoretical and Applied Genetics* **88**, 933–944.

Allaby, R.G. and Brown, T.A. (2003) AFLP data and the origins of domesticated crops. *Genome* **46**, 448–453.

Bewley, J.D. (1997) Seed germination and dormancy. *Plant Cell* **9**, 1055–1066.

Biss, P., Freeland, J., Silvertown, J., McConway, K. and Lutman, P. (2003) Successful amplification of rice chloroplast microsatellites from century-old grass samples from the Park Grass experiment. *Plant Molecular Biology Reporter* **21**, 249–257.

Bowcock, A.M., Ruiz-Linares, A., Tomfohrde, J., Minch, E., Kidd, J.R. and Cavalli-Sforza, L.L. (1994) High resolution of human evolutionary trees with polymorphic microsatellites. *Nature* **368**, 455–457.

Bromham, L. and Penny, D. (2003) The modern molecular clock. *Nature Reviews Genetics* **4**, 216–224.

Brown, T.A. (1999) How ancient DNA may help in understanding the origin and spread of agriculture. *Philosophical Transactions of the Royal Society of London, Series B* **354**, 89–98.

Cheng, S., Fockler, C., Barnes, W.M. and Higuchi, R. (1994) Effective amplification of long targets from cloned inserts and human genomic DNA. *Proceedings of the National Academy of Sciences, USA* **91**, 5695–5699.

Chin, E.C.L., Senior, M.L., Shu, H. and Smith, J.S.C. (1996) Maize simple repetitive DNA sequences: Abundance and allele variation. *Genome* **39**, 866–873.

Colosi, J.C. and Schaal, B.A. (1997) Wild proso millet (*Panicum miliaceum*) is genetically variable and distinct from crop varieties of proso millet. *Weed Science* **45**, 509–518.

Cooper, A. and Poinar, H.N. (2000) Ancient DNA: Do it right or not at all. *Science* **289**, 1139.

Cooper, A., Lalueza-Fox, C., Anderson, S., Rambaut, A., Austin, J. and Ward, R. (2001) Complete mitochondrial genome sequences of two extinct moas clarify ratite evolution. *Nature* **409**, 704–707.

Cresswell, A., Sackville-Hamilton, N.R., Roy, A.K. and Viegas, B.M.F. (2001) Use of AFLP markers to assess genetic diversity of *Lolium* species from Portugal. *Molecular Ecology* **10**, 229–241.

Deguilloux, M.-F., Pemonge, M.-H. and Petit, R.J. (2002) Novel perspectives in wood certification and forensics: dry wood as a source of DNA. *Proceedings of the Royal Society of London, Series B* **269**, 1039–1046.

Gorman, C.F. (1969) Hoabinhian: a pebble-tool complex with early plant associations in Southeast Asia. *Science* **163**, 671–673.

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

Gyulai, F. (2000) *Seed and fruit collections of the middle European plant species*. Godollo, Hungary, St. Stephanus University.

Gyulai, G., Mester, Z., Kiss, J., Szeman, L., Heszky, L. and Idnurm, A. (2003) Somaclone breeding of reed canarygrass (*Phalaris arundinacea* L). *Grass and Forage Science* **58**, 210–215.

Gyulai, G., Humphreys, M., Bittsánszky, A., Skot, K., Kiss, J., Skot, L., Gullner, G., Heywood, S., Szabo, Z., Lovatt, A., Radimszky, L., Roderick, H., Rennenberg, H., Abberton, M., Kőmíves, T. and Heszky, L. (2005) AFLP analysis and improved phytoextraction capacity of transgenic *gsh*I-poplar clones (*Populus canescens* L.) *in vitro*. *Zeitschrift für Naturforschung* **60c**, 300–306.

Harlan, J.R. (1971) Agricultural origins: centers and noncenters. *Science* **174**, 468–473.

Ho, P.-T. (1977) The indigenous origins of Chinese agriculture. pp. 413–418 *in* Reed, C.A. (Ed.) *Origins of agriculture*. Paris, Mouton Publishers.

Hofreiter, M., Jaenicke, V., Serre, D., von Haeseler, A. and Pääbo, S. (2001) DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. *Nucleic Acids Research* **29**, 4793–4799.

Keng, H. (1974) Economic plants of ancient north China as mentioned in *Shih Ching* (Book of Poetry). *Economic Botany* **28**, 391–410.

Lagler, R., Gyulai, G., Humphreys, M., Szabo, Z., Horvath, L., Bittsanszky, A., Kiss, J., Holly, L. and Heszky, L. (2005) Morphological and molecular analysis of common millet (*P. miliaceum*) cultivars compared to an aDNA sample from the 15th century (Hungary). *Euphytica* **146**, 77–85.

Michelmore, R.W., Paran, I. and Kesseli, R.V. (1991) Identification of markers linked to disease-resistance genes by bulked-segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proceedings of the National Academy of Sciences, USA* **88**, 9828–9832.

Nyekhelyi, B.D. (2003) *Monumenta historica Budapestinensia XII*. Hungary, Historical Museum of Budapest.

Pääbo, S., Poinar, H., Serre, D., Jaenicke-Despres, V., Hebler, J., Rohland, N., Kuch, M., Krause, J., Vigilant, L. and Hofreiter, M. (2004) Genetic analyses from ancient DNA. *Annual Review of Genetics* **38**, 645–679.

Petit, R.J., Demesure, B. and Dumolin, S. (1998) cpDNA and mtDNA primers in plants. pp. 256–261 *in* Karp, A.; Isaac, P.G.; Ingram, D.S. (Eds) *Molecular tools for screening biodiversity*. London, Chapman & Hall.

Poinar, H.N. and Stankiewicz, B.A. (1999) Protein preservation and DNA retrieval from ancient tissues. *Proceedings of the National Academy of Sciences, USA* **96**, 8426–8431.

Poinar, H.N., Kuch, M., McDonald, G., Martin, P. and Pääbo, S. (2003) Nuclear gene sequences from a late Pleistocene sloth coprolite. *Current Biology* **12**, 1150–1152.

Priestley, D.A. (1986) *Seed aging: Implication for seed storage and persistence in the soil*. Ithaca, New York, Cornell University Press.

Raniello, R. and Procaccini, G. (2002) Ancient DNA in the seagrass *Posidonia oceanica*. *Marine Ecology – Progress Series* **227**, 269–273.

Roder, M.S., Korzun, V., Wendehake, K., Plaschke, J., Tixier, M.H., Leroy, P. and Ganal, M.W. (1998) A microsatellite map of wheat. *Genetics* **149**, 2007–2023.

Saltonstall, K. (2003) Microsatellite variation within and among North American lineages of *Phragmites australis*. *Molecular Ecology* **12**, 1689–1702.

Schermann, Sz. (1966) *Magismeret, Vols I and II* (Seed Atlas, in Hungarian). Budapest, Akadémiai Kiado.

Skøt, L., Hamilton, N.R.S., Mizen, S., Chorlton, K.H. and Thomas, I.D. (2002) Molecular genecology of temperature response in *Lolium perenne*: 2. Association of AFLP markers with ecogeography. *Molecular Ecology* **11**, 1865–1876.

Smith, P.M. (1976) Minor crops. pp. 301–324 *in* Simmonds, N.W. (Ed.) *Evolution of crop plants*. London, Longman.

Sun, G., Kaushal, R., Pal, P., Wolujewicz, M., Smelser, D., Cheng, H., Lu, M., Chakraborty, R., Jin, L. and Deka, R. (2005) Whole-genome amplification: relative efficiencies of the current methods. *Legal Medicine* **7**, 279–286.

Szabo, Z., Gyulai, G., Humphreys, M., Horváth, L., Bittsánszky, A., Lagler, R. and Heszky, L. (2005) Genetic variation of melon (*C. melo*) compared to an extinct landrace from the Middle Ages (Hungary) I. rDNA, SSR and SNP analysis of 47 cultivars. *Euphytica* **146**, 87–94.

Threadgold, J. and Brown, T.A. (2003) Degradation of DNA in artificially charred wheat seeds. *Journal of Archaeological Science* **30**, 1067–1076.

Toth, G., Gaspari, Z. and Jurka, J. (2000) Microsatellites in different eukaryotic genomes: survey and analysis. *Genome Research* **10**, 967–981.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Friters, A., Pot, J., Paleman, J., Kuiper, M. and Zabeau, M. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407–4414.

Walters, T.W. (1989) Historical overview on domesticated plants in China with special emphasis on the *Cucurbitaceae*. *Economic Botany* **43**, 297–313.

Willerslev, E., Hansen, A.J., Binladen, J., Brand, T.B., Gilbert, M.T.P., Shapiro, B., Bunce, M., Wiuf, C., Gilichinsky, D.A. and Cooper, A. (2003) Diverse plant and animal genetic records from Holocene and Pleistocene sediments. *Science* **300**, 791–795.

Yang, H. (1997) Ancient DNA from Pleistocene fossils: preservation, recovery, and utility of ancient genetic information for quaternary research. *Quaternary Science Reviews* **16**, 1145–1161.

Received 17 October 2005
accepted after revision 2 June 2006