Euphytica (2005) 146: 77–85
DOI: 10.1007/s10681-005-5814-7

Morphological and molecular analysis of common millet (*P. miliaceum*) cultivars compared to an aDNA sample from the 15th century (Hungary)

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Received 30 November 2004; accepted 20 April 2005

Key words: ancientDNA, CAP, mtDNA, ISSR, SNPs

Summary

Morphological characterization of 20 common millet (*Panicum miliaceum* L., $2n = 4x = 36$) cultivars and landraces revealed four distinct clusters which were apparently consistent with the grain colors of black, black and brown, red, yellow, and white. Seed remains of medieval millet, recovered from a 15th century layer (King's Palace, Budapest, Hungary), showed reddish yellow grain color after rehydrating on tissue culture medium that was close to grain color of modern cultivar *Omszkoje*. aDNA of medieval commom millet was extracted successfully, analyzed and compared to modern common millets by ISSR, SSR, CAP and mtDNA. Analyses of fragments and sequences revealed polymorphism at seven ISSR loci (15 alleles) and at the 5S-18S rDNA locus of mtDNA. CAP analysis of the 5S-18S rDNA fragment revealed no SNPs in the restriction sites of six endonucleases *Taq*I, *Bsu*RI, *Hinf*I, *Mbo*I, *Alu*I and *Rsa*I. Sequence alignments of the restriction fragments *Rsa*I also revealed consensus sequence in the medieval sample compared to a modern variety. An attempted phenotype reconstruction indicated that medieval common millet showed the closest morphological similarity to modern millet cultivar *Omszkoje*.

Introduction

Genes recovered from aDNA of extinct species and varieties supplies new data for evaluate changes in genetic variation that occurred during microevolution of cultivated plants over the past hundreds of years (Aufhammer & Fischbeck, 1964; Ruckenbauer, 1971; Suh et al., 2000). These genes, after gene rescue and cloning, could supply useful alleles for broadening genetic variation of cultivated plants.

Several extinct varieties of crops have been reintroduced into breeding programs by re-germination of aged seeds such as a 127 year old hexaploid Hungarian wheat (Székesfehérvári – Stuhlweissenburger) (*Triticum vulgare* var. *erythrospermum* Körn.) recovered from 1877 in Vienna (Ruckenbauer, 1971); a 172 year old barley (*Hordeum*) and oat (*Avena*) recovered in Nürnberg (Aufhammer & Fischbeck, 1964); and the questionable Egyptian wheat *Kamut* (*T. turanicum*) (Quinn, 1999; Grausgruber et al., 2004). None of these programs included DNA analysis.

In the present study analysis of aDNA extracted from medieval (15th century) common millet (*P. miliaceum*) is reported. Molecular analysis was carried out by SSR (simple sequence repeat), ISSR (inter-simple sequence repeat), mtDNA (mitochondrial DNA) and CAP (cleaved amplified polymorphic DNA), prior to morphological characterization with 20 modern cultrivars and landraces with a final aim of cultivar reconstruction of a 600 years old common millet.

Materials and methods

Seed samples

Grain remains of common millet (*P. miliaceum*) were excavated from the 15th century layer at King's Palace of Budapest, Buda Hill (Hungary) (Nyékhelyi, 2003). 150 medieval grains (#21, Figure 1b) were sorted and identified in the laboratory, followed by surface sterilization (8%, NaOCl) and 3 months incubation on aseptic tissue culture medium F6 (Gyulai et al., 2003).

Morphological characterization

Twenty modern cultivars (Table 1) were grown up in 5 m × 5 m plots in duplicate experiments, and characterized morphologically (Figure 1) according to the standards of Descriptor Lists of the ABI (AgroBotanical Institute, Tápiószele, Hungary) and the National Institute for Quality Control (OMMI, 2004).

In total, 24 traits were recorded such as (1) seedling anthocyanin (absent, weak or strong), (2) leaf anthocyanin (absent, weak or strong), (3) stigma anthocyanin (absent, weak or strong), (4) seedling pubescence (absent, medium, dense or very dense), (5) plant pubescence (naked, sparse, dense), (6) leaf length (mm), (7) leaf width (mm), (8) leaf number per plant, (9) stem number per plant, (10) stem thickness (mm), (11) plant branching habit (erected, semi-loose, loose), (12) glume color (light-green dark-green or with anthocyanin), (13) heading date (numbers of days), (14) panicle shape (spreading and open, loose and one-sided or compact and erect), (15) panicle bending (weak, medium or strong), (16) panicle length (cm), (17) plant height (m), (18) seed shape (elongated, oval or rounded), (19) seed color (white, cream, yellow, red, brown, grey, black) (Figure 1), (20) color of endosperm (amber-yellow, yellow or whitish-yellow), (21) seed weight (1000 seeds), (22) lodging (%), (23) uniformity (variegated, medium or uniform), (24) *Xanthomonas*

Table 1. Common millet (*P. miliaceum)* cultivars, landraces (lr) (ABI, Tápiószele, Hungary) and medieval sample characterized with DNA data observed

#	Cultivars/ landraces	Short name	Grain color	Accession #	Provided		DNA (concentration ng/μl; and absorption data)					
					Country	Date	ng/μl	230 nm	260 nm	280 nm	260/280	260/230
1	*Tápiószele-D*	TAPD	Black	RCAT073416	POL	1999	301,44	3,382	6,177	3,279	1,93	1,86
2	*Tápiószele-C*	TAPC	Black & brown	RCAT073585	ITA	1999	375,99	4,761	7,911	4,321	1,91	1,72
3	*Tápiói barna*	TABA	Black & brown	RCAT017521	HUN	1988	235,42	2,852	4,861	2,596	1,93	1,74
4	*Tápiószentmártoni (lr)*	DEBR	Black & brown	RCAT017513	HUN	1987	707,68	17,04	18,576	12,31	1,79	1,12
5	*Tápiószele-B*	TASZ	Brown	00185/01	HUN	2001	417,55	5,869	9,183	5,267	1,88	1,66
6	*Debreceni barna*	DEBA	Brown	RCAT017280	HUN	1957	299,21	3,894	6,387	3,532	1,91	1,71
7	*Fertődi 10.D*	FERT	Grey	RCAT017272	HUN	1960	336,69	4,01	6,933	3,722	1,91	1,77
8	*Püski (lr)*	PÜSK	Grey	RCAT017296	HUN	1959	322,38	4,77	7,252	4,274	1,86	1,63
9	*Rábaszentandrási (lr)*	RABA	Grey	RCAT017297	HUN	1954	321,18	5,163	7,531	4,568	1,86	1,58
10	*Bolgár-159*	BOLG	Grey	RCAT017267	HUN	1954	306,87	3,808	6,507	3,552	1,93	1,79
11	*Fertődi piros*	FEPI	Red	RCAT017291	HUN	1960	334,58	8,937	9,869	7,096	1,71	1,16
12	*Kecskeméti (lr)*	KEKE	Red	RCAT017527	HUN	1986	218,98	2,559	4,572	2,481	1,91	1,85
13	*Omszkoje*	OMOS	Red & yellow	02546/00	RUS	2000	304,70	3,939	6,554	3,645	1,91	1,75
14	*Jászberényi (lr)*	JASZ	Yellow	RCAT017555	HUN	1977	339,45	6,28	8,306	5,229	1,83	1,43
15	*Császárréti*	CSAS	Yellow	RCAT017277	HUN	1960	321,48	7,157	8,564	5,804	1,75	1,28
16	*Nyiregyházi (lr)*	NYIR	Yellow	RCAT017526	HUN	1987	271,20	3,757	5,826	3,217	1,93	1,62
17	*Tápiószele-A*	TAPA	Yellow	RCAT017509	SUN	1985	199,66	2,539	4,245	2,353	1,9	1,75
18	*Fertődi fehér*	FEFE	Yellow	RCAT017290	HUN	1960	332,83	6,454	8,457	5,493	1,8	1,43
19	*Martonvásári-3*	MART	Yellow	RCAT017285	HUN	1957	722,44	12,34	16,9	10,356	1,83	1,46
20	*Mesterházi (lr)*	MEST	White	RCAT017494	HUN	1982	275,54	4,727	5,928	3,314	1,9	1,28
21	15th century millet	15.c	Reddish yellow	–	HUN	2003	8,82	0,264	0,244	0,172	1,68	0,90

HUN: Hungary, ITA: Italy, POL: Poland and RUS: Russia.

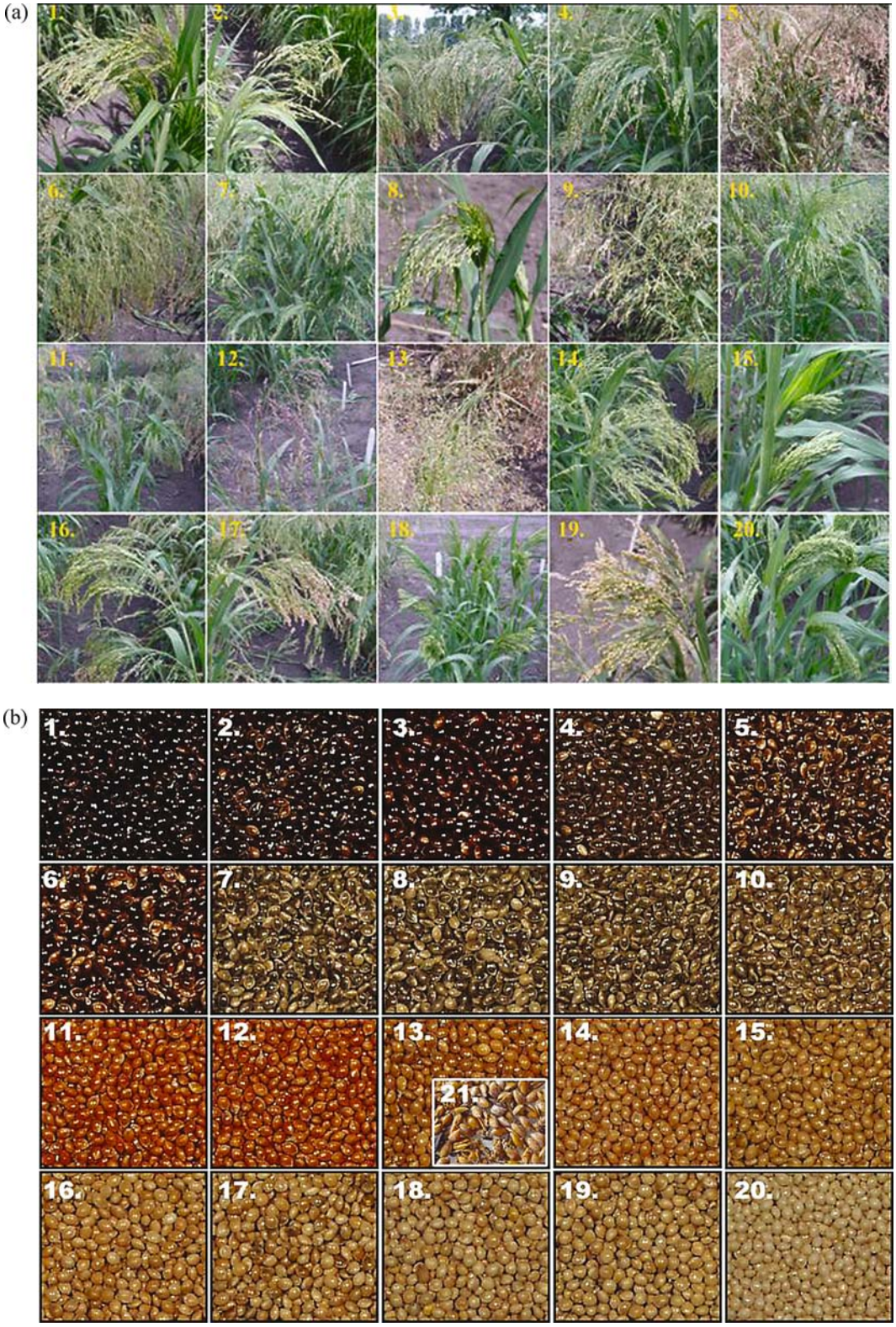

Figure 1. Types of panicles (**a**) and grains (**b**) (1–20) including 15th century grains (21) of common millet (*P. miliaceum*) (Table 1) investigated.

infection (absent, low, medium, high, extremely high). Photos were taken with a digital camera.

DNA extraction

Total aDNA of 78 aseptic medieval seed remains and seed DNA of modern cultivars (0.1 g) in each case was extracted in CTAB, cethyltrimethylammonium bromide, buffer (Murray & Thompson, 1980; Doyle & Doyle, 1990) followed by an RNase-A treatment (Sigma, R-4875) for 30 min at 37 °C. The quality and quantity of extracted DNA were measured by a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Delaware, USA – Bio-Science, Budapest, Hungary). DNA samples were adjusted to concentration of 30 ng/μl with ddH_2O and subjected to PCR amplification.

ISSR analysis

For ISSR analysis the basic protocol of Zietkiewitz et al. (1994) was applies using nine primers and combinations: (1) FV808 – $(ag)_8$c; (2) FV810 – $(ga)_8$t; (3) FV811 – $(ga)_8$c; (4) FV819 – $(gt)_8$a; (5) FV820 – $(gt)_8$c; (6) FV821 – $(gt)_8$t; (7) FV835 – $(ag)_8$(t/c)c; (8) FV836 – $(ag)_8$(t/c)g and (9) FV841 – $(ga)_8$(t/c)c according to Cekic et al. (2001).

CAP and mtDNA analysis

Mitochondrial DNA at 5S-18S rDNA of mtDNA locus was amplified by primer the pair 5′-gtg ttg ctg aga cat gcg cc-3′ and 3′-ata tgg cgc aag acg att cc-5′ according to Petit et al. (1998). Fragments were purified from the EtBr-stained (0.5 ng/μl) agarose (0.8%) gel by a spin column (Sigma G-6501) and digested with seven restriction endonucleases (REs) *Taq*I, *Bsu*RI, *Hinf*I, *Mbo*I, *Alu*I and *Rsa*I (2 μl) following the manufacturer's protocol (Fermentas). Digested DNA samples (14 μl) were separated on 1.6% agarose gel, and photographed under a transilluminator. *Rsa*I restriction fragments of the medieval sample and cv. *Omszkoje* (#13) was purified and sequenced.

Sequencing

Fragments were isolated from the agarose gel by a spin column (Sigma, 5-6501) and subjected to automated fluorescent DNA sequencing (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Hungary). Sequences were analyzed by the computer program ChromasPro version 1.11 (Technelysium Pty Ltd, USA). Alignments of sequences were analyzed by BioEdit Sequence Alignment Editor (NCSU, USA). BLAST (Basic Local Alignment Search Tool) analysis was carried out by the computer program of NCBI (National Center for Biotechnology Information).

Data analysis

Cluster analysis based on presence versus absence of fragments was carried out by SPSS-11 using Jaccard's coefficient (Jaccard, 1908) with average distance between groups.

Results

Morphological analysis

Modern common millet cultivars and landraces (1 to 20) included the three main panicle forms of spreading and open; loose and one-sided; and compact and erected (Figure 1a). Grain colors varied from black, black and brown, brown, grey, red, yellow, cream and white (Figure 1b).

The cluster analysis based on the inspected 24 morphological characters grouped the modern cultivars into four distinct clusters which were consistent with the seed colors (Figure 2).

Molecular analysis

aDNA sample extracted from the 78 aseptic grains of 150 seed remains (# 21) (Figure 1) were grouped into DNA pools according to Michelmore et al. (1991).

In the ISSR analysis, seven of the nine ISSR primers (Cekic et al., 2001) and combinations amplified 15 alleles in the medieval sample and modern millet cultivars (Figure 3). The sequences of ISSR fragments amplified by the combinations of primer FV810 – FV841 showed identical origin of common millet with no SNPs (Figure 4).

The mitochondrial specific primer pair amplified an 1117 bp fragment at the 5S-18S rDNA locus of mtDNA in the medieval millet and modern millet *Omszkoje* (Figure 5, 1 & 2).

In the CAP-analysis of this fragment there were no observed changes in the DNA sequences at restriction sites of *Taq*I, *Bsu*RI, *Hinf*I, *Mbo*I, *Alu*I and *Rsa*I giving the same fragment patterns on agarose

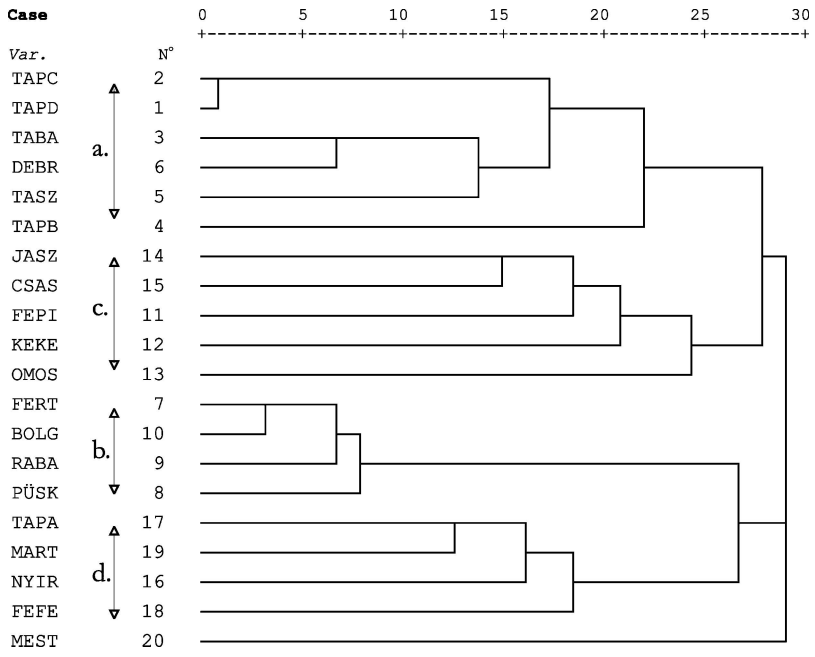

Figure 2. Cluster analysis of common millet (*P. miliaceum*) cultivars and landraces (1–20) (Table 1). Arrows indicate clusters a–d.

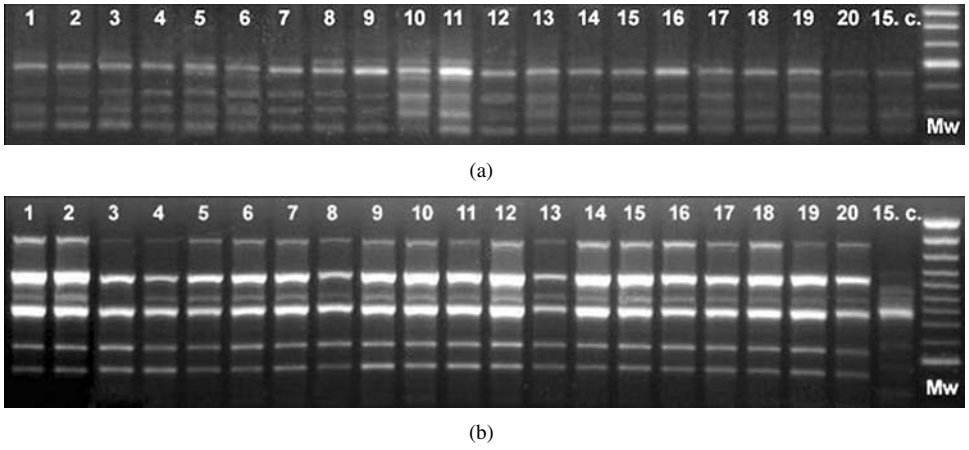

Figure 3. Samples of ISSR analysis with monomorphic (a) and polymorphic (b) patterns on agarose gel (0.8%) amplified by primers of FV835 – $(ag)_8$yc (a) and FV811 – $(ga)_8$c (b) in the modern common millet (*P. miliaceum*) cultivars and landraces (1–20) (Table 1) compared to medieval common millet sample (15.c). Mw – 100 bp DNA ladder.

gel with the same numbers and length of digested fragments (Figure 5). After sequencing of *Rsa*I fragments no SNPs were observed in the sequences (Figure 6).

Discussion

Common (syn: broomcorn-, hog-, hershey-, yellow- and white-) millet *(P. miliaceum*) as one of the most ancient cereal crop is a self-pollinated, short-day, C4 photosynthetic plant with 55% harvest index. Plant height is about 30 to 100 cm with extreme short ripening time (60 to 75 days) and the lowest water requirement of any grain crops. Seed size is about 3 mm long and 2 mm wide with variation in color from white to black (Figure 1). Spikelets of *Panicum* have 2 flowers, the lower floret is sterile and the upper is fertile and hardened. Similar to all monocots, millet

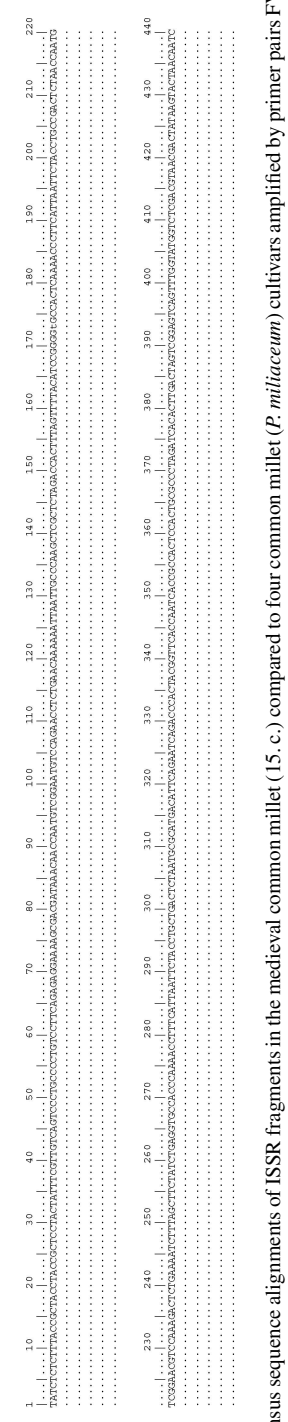

Figure 4. Consensus sequence alignments of ISSR fragments in the medieval common millet (15. c.) compared to four common millet (*P. miliaceum*) cultivars amplified by primer pairs FV810 – $(ga)_8$t and FV841 – $(ga)_8$(t/c)c.

'seed' is a *caryopsis* which is enclosed within a hard shiny hull (lemma and palea).

The oldest historical reports of common millet ('*su*') are from China 5000–3200 B.C., where field agriculture and animal domestication apparently begun (Harlan, 1975; Ho, 1977). In China common millet was cultivated together with foxtail millet (*P. italicum*) as the main crops (Ho, 1969; Walters, 1989). In the ancient Chinese 'Book of Poetry' (Shih Ching), written about 1000–500 B.C., nine poems sing about common millet ('*shu*' or '*tsi*'). Later, *Panicum* became typical foods of the Sumer and Northern India together with barley (*Hordeum vulgare*) in about 2500 B.C. For the nations of steppic Scythia as Celtics or Hungarians the common millet was the first crop 2000 B.C.

As the most drought-resistant crop with extremely short ripening time, common millet was optimal for the semi-nomadic tribes living in the Steppes making two harvests in 1 year. Common millet spread from the Steppes through Europe via tribes of the Celts, Huns, Avars and Hungarians, and also through the region of 'Fertile Crescent' and Africa. It was the *milium* of Romans (Smith, 1977).

Recently common millet is frequently cultivated in warm temperate and sub-tropical zones as a late-seeded, short-season summer catch crop with several cultivars. Nevertheless, as a species of the group of small millets including finger millet (*Eleusine coracana*), foxtail millet (*Setaria italica*), kodo millet (*Paspalum scrobiculatum*), little millet (*Panicum sumatrense*) and barnyard or sawa millets (*Echinochloa crus-galli* and *Echinochloa corona*), it accounts for less than 1% of the food grains produced in the world today (Chang, 1968; Baltensperger et al., 1997). The new fashion to grow common millet is not only as an alternative crop, or bird food crop, but is also cultivated for its unique nutritional value which is superior to main cereals of wheat, rice, oats, etc. *Panicum* flour is gluten-free and more easily digestible because of it's high alkaline content which counteracts acids, and high protein (10–15%) content (Chang, 1968; Geervani & Eggum, 1989). Some new cultivars such as '*Earlybird*', '*Sunrise*' and '*Hunstman*' and new types with waxy starch were registered recently (Baltensperger et al., 1997).

Morphological analysis

The shape and structure of panicles comprise three main types in common millet (Colosi & Schaal, 1997; Scholz & Mikolas, 1991). The 20 studied cultivars in-

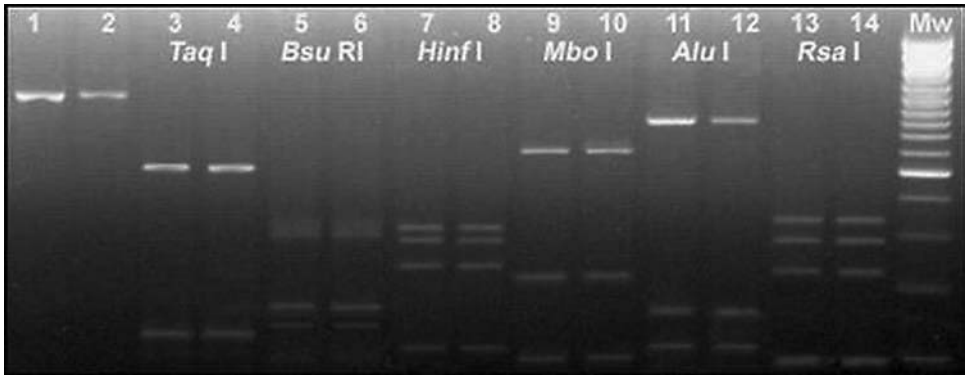

Figure 5. CAP analysis of mtDNA fragments at 5S-18S rDNA locus in the medieval common millet (*P. miliaceum*) (1, 3, 5, 7, 9, 11, 13) and modern cultivar *Omszkoje* (2, 4, 6, 8, 10, 12, 14) after digestion by REs *Taq*I, *Bsu*RI, *Hinf*I, *Mbo*I, *Alu*I and *Rsa*I. Mw – 100 bp DNA ladder.

cluded all these types: the open panicle (# of 4 to 6, 9 and 13), loose and one-sided (# of 2, 3, 7, 12–14, and 16–17); and compact panicle (# of 1, 8, 18 and 18–20). Panicle types combine with different grain colors from black to white (Figure 1).

Dendrogram based on morphological characters grouped the cultivars (1 to 20) into four distinct clusters which were apparently consistent with the grain colors of black (1 to 6); black and brown (7 to 10); red (11–12); yellow including two clusters (13 to 15) and (17 to 19); and white (20) but not striped (Figure 2). Medieval grain showed reddish yellow color after rehydration on tissue culture medium that was close to grain color of the modern varieties 11 to 15 (*Fertődi piros* – 11, *Kecskeméti*-lr – 12, *Jászberényi*-lr – 14 and *Császárréti* – 15) including *Omszkoje* – 13 (Figure 1). This group of modern cultivars was included in the molecular analysis.

Molecular analysis

The famous excavations (Nyékhelyi, 2003) from the 15th century layer at the King's Palace of Budapest, Buda Hill (Hungary) revealed about 3 million of plant remains of 196 plant species, including 955497 seed of common millet (detailed elsewhere). For the study presented here 150 grains of common millet (*P. miliaceum*) were selected which showed good preservation due to the anaerobic conditions in the slime of a deep medieval well covered by water (Figure 1).

For safe aDNA isolation 150 medieval grains were surface sterilized and incubated on aseptic conditions to exclude all the fungal or bacterial infected seeds.

The quality and quantity of pooled aDNA extracted from 78 aseptic medieval grains was measured by NanoDrop ND-1000 UV-Vis spectrophotometer, which enabled highly accurate analyses of extremely small samples (2 μl DNA) with remarkable reproducibility. The quantity of aDNA (8.82 ng/μl) was significantly less then DNA samples of modern cultivars, nevertheless aDNA showed good spectral quality (Table 1) with apparent degradation in the high molecular weight fragments as observed on agarose gel compared to modern cultivars (not shown).

The CTAB method applied in the present study was found to be the most reliable method for isolation aDNA in an SSR analysis of century-old grass samples (leaves) of *Anthoxanthum odoratum* and *Festuca rubra* with compared to five different extraction protocols (Biss et al., 2003).

Seven of the nine ISSR primers amplified fragments in the medieval and modern millets (Figure 3) and only two FV819 – $(gt)_8$a and FV820 – $(gt)_8$c failed amplification. In total 15 ISSR alleles were detected. A molecular cluster based on the presence versus absence of ISSR fragments indicated that medieval common millet showed the closest genetic similarity to a Russian registered modern variety *Omszkoje* (# 13) (not shown). Unexpectedly, no SNPs were detected in the sequences of ISSR fragments (Figure 4), which might indicate the applicability of ISSR for the detection of conserved DNA regions (Zietkiewicz et al., 1994). ISSR was also reported to reveal highly conserved sequences between species of *Arabidopsis thaliana* and *Brassica oleracea* var. *botrytis* (Bornet et al., 2002). Nevertheless, dominant markers such as ISSR was found to be less suitable for genotyping aDNA because the absence of a fragment could be either due to the loss

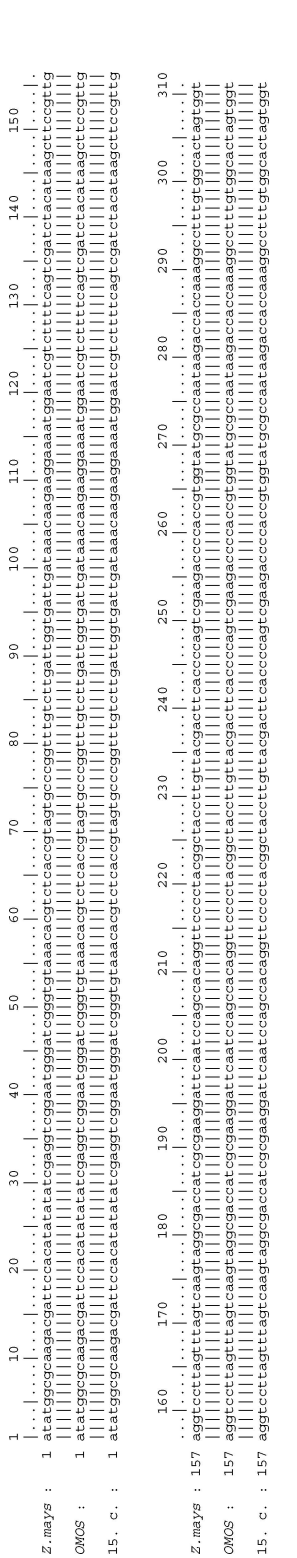

Figure 6. Consensus sequence alignments of *Rsa*I fragments (312 bp) of mtDNA at the 18S-5S-rDNA locus in the medieval common millet (*P. miliaceum*) (15. c.) compared to modern common millet cultivar *Omszkoje* and NCBI database (*Z. mays*, X00794).

of the degraded DNA sequence or the variation of that locus in the genome (Biss et al., 2003). However, ISSR in the present study was highly useful in isolating sequences from a aDNA sample of common millet (Figure 4).

Consensus sequences at the 5S-18S rDNA (1117 bp) locus of mtDNA, and at six restriction sites (*Taq*I, *Bsu*RI, *Hinf*I, *Mbo*I, *Alu*I and *Rsa*I) of this fragment (CAP) were also detected (Figure 5). Sequence analysis of *Rsa*I fragment also showed complete sequence homology between medieval common millet and cv. *Omszkoje* (# 13) (Figure 6). All these data indicate both the evolutionary highly conserved sequences detected at mtDNA and ISSRs (Al-Jabani et al., 1994), and the relatively short time of 600 years for microevolution and mutations of SNPs in common millet (Parani et al., 2001).

In contrast, RAPD polymorphism clustered common millets accessions according to geographical regions of origin, which indicated that genome of common millet was fairly plastic with potential for relatively rapid adaptation period during microevolution (M'Ribu & Hilu, 1994). An olive-black seeded biotype called 'wild proso millet' which appeared in North America 1970, after about 400 years of millet cultivation (as millet was introduced to North America in the 17th century) also indicated a plastic common millet genome (Strand & Behrens, 1981). Weedy biotypes of common millet were also reported from Europe (Scholz & Mikolas, 1991).

To conclude, morphological and aDNA analysis of grains of medieval and modern common millets (*P. miliaceum*) revealed that medieval millet showed the closest similarity to a Russian registered modern cultivar *Omszkoje* (Figure 1, # 13) which might provide an insight to reconstruct a medieval common millet recovered from the 15th century.

Acknowledgments

The project was sponsored by grants of OTKA-M-04563, FVM-98857/14/2003, FVM-69757, Bio-342/2001, OECD-JA00018642 and SzÖ-4/2003. Seed remains from the Middle Ages were excavated and kindly supplied by Prof. Ferenc Gyulai.

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