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Spelt-specific alleles in HMW glutenin genes from modern and historical European spelt (*Triticum spelta* L.)

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Abstract A partial promoter region of the high-molecular weight (HMW) glutenin genes was studied in two wheat specimens, a 300 year-old spelt (*Triticum spelta* L.) and an approximately 250 year-old bread wheat (*Triticum aestivum* L.) from Switzerland. Sequences were compared to a recent Swiss landrace *T. spelta* 'Oberkulmer.' The alleles from the historical bread wheat were most similar to those of modern *T. aestivum* cultivars, whereas in the historical and the recent spelt specific alleles were detected. Pairwise genetic distances up to 0.03 within 200 bp from the HMW Glu-A1-2, Glu-B1-1 and Glu-B1-2 alleles in spelt to the most-similar alleles from bread wheat suggest a polyphyletic origin. The spelt Glu-B1-1 allele, which was unlike the corresponding alleles in bread wheat, was closer related to an allele found in tetraploid wheat cultivars. The results are discussed in context of the origin of European spelt.

Keywords Wheat · *Triticum spelta* L. · HMW glutenin · Ancient DNA · Evolution

Introduction

Spelt (*Triticum spelta* L.) is a hexaploid wheat (genomes AABBDD) characterized morphologically by lax spikes, a semi-tough rachis and tough, adhering glumes (Lupton 1987). Whereas einkorn (*Triticum monococcum* L.), emmer (*Triticum dicoccum* (Schrank.) Schübl.) and the free-theshing wheats (*Triticum aestivum*/*durum*/*turgidum*) (Hillman et al. 1996) are consistently present in the European archaeological record since Neolithic times (Jacomet and Kreuz 1999), there is very little evidence for spelt in Europe before the Bronze Age. From at least the late Bronze Age onwards, spelt was of regional importance in several parts of Europe (e.g. Nesbitt and Samuel 1996), especially in mountainous regions like the northern Alpine area (Percival 1974). Free-threshing hexaploid bread wheat (*T. aestivum* L.) and spelt share the same genomes (AABBDD), but their relation remains controversial. Two main hypotheses have been suggested for the origin of European spelt.

Earlier in the last century several authors (e.g. Bertsch 1943; Flaksberger 1930) postulated that spelt emerged by a hybridization of a hulled tetraploid emmer and free-threshing hexaploid wheat in Europe. This possibility was confirmed experimentally by crossing emmer and bread wheat, resulting in a spelt-like alloploid (MacKey 1966). However, archaeobotanical data suggest that while emmer was widespread in the Bronze Age, evidence for the presence of bread wheat in Central Europe in this period is rare (Maier 1996; Schlumbaum et al. 1998).

The alternative hypothesis proposed that spelt originated from the cross of a hulled tetraploid wheat, e.g. *T. dicoccum* (genomes AABB), with wild *Aegilops tauschii* Coss. (genomes DD), growing in the southern areas of the Caspian Sea (McFadden and Sears 1946). In this model *T. aestivum* was derived from spelt by few mutations (Sears 1974) and both wheats subsequently reached Europe with the spread of agriculture (Zohary and Hopf 2000). An Asian origin of spelt was further supported by the discovery of spelt-cultivation in Iran (Kuckuck and Schiemann 1957). However, the absence of spelt at archaeological sites in the Near East is not consistent with this hypothesis (for review Nesbitt and Samuel 1996).

In recent years aspects of both hypotheses have been tested using molecular markers. Whereas the origin of the D-genome in *T. aestivum* from *Ae. tauschii* in the Near East has been confirmed (Dvorák et al. 1998a), it is increasingly doubtful whether today's European spelts are directly descended from the spelt-like ancestors of bread wheat. European spelt seems genetically more dis-

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tant from *T. aestivum* than Asian spelt (Dvorák et al. 1998b), and European and Asian spelt differ in genes controlling spike morphology (Luo et al. 2000). Other studies showed that European spelt and bread wheat vary by more than a few genes responsible for the brittleness of the rachis and the glumes (Liu and Tsunewaki 1991; Siedler et al. 1994).

Historical wheat-remains offer important advantages to further investigate the history of hexaploid wheat in Europe. They represent landraces grown before the onset of industrial breeding programs and the international movement of seed stocks. Historical wheat-remains up to several hundred years old were used as insulation material in ceilings of historical buildings, e.g. in Switzerland, and were preserved desiccated. The analysis of DNA derived from desiccated plant material was reported previously from herbarium material (Savolainen et al. 1995) and from archaeological remains (O'Donoghue et al. 1996; Rollo et al. 1987; Deakin et al. 1998).

Genes encoding seed storage proteins such as the glutenins are among the best-studied genes in wheat. The Glu-1 loci are located on chromosome 1A, 1B and 1D in hexaploid wheat. Each consists of two genes, encoding one x-type and one y-type HMW glutenin subunit (Payne 1987). The genes are genome-specific and multiallelic. A phylogenetic analysis of a 240-bp fragment of the HMW glutenin promoter region (further HGP) was consistent with the current understanding of wheat evolution (Allaby et al. 1999). The fragment was also used in previous studies on ancient DNA from archaeological wheat (Allaby et al. 1994; Schlumbaum et al. 1998).

Here we report the analysis of a 240-bp fragment of the HGP from desiccated chaff of spelt (*Triticum spelta* L.) and bread wheat (*T. aestivum* L.), retrieved from the ceilings of two historical buildings (300 and approximately 250 years old, respectively) in the region of Basel, Switzerland. A recent landrace of spelt was analysed as a reference and the results were compared to published HGP sequences.

Materials and methods

Plant materials

Single spikelets of *T. spelta* L. were recovered from undisturbed context during renovations of a 300 year-old mill in Arlesheim (Kanton Baselland, Switzerland). A complete rachis with some spikelets of *T. aestivum* L. was found in an over 400 year-old farmhouse in Itingen (Kanton Baselland). The age of the sample was estimated to be about 250 years, when major reconstruction took place. Both samples consisted of chaff derived from cereal processing and were used as insulation material in the ceilings of the buildings. They were desiccated and contained no grains (Fig. 1). Identification was based on morphology (Jacomet et al. 1989).

T. spelta L. 'Oberkulmer' (TsOb) was provided by B. Keller (Universität Zürich, CH). TsOb is a selection of a Swiss landrace, collected in the 1930s in Oberkulm (Kanton Aargau) (Weilenmann, personal communication), located near Arlesheim (43 km) and Itingen (32 km). TsOb is an important parent to many modern spelt accessions (Siedler et al. 1994; Bertin et al. 2001).

Fig. 1 Desiccated 300 year-old empty spikelets from *T. spelta* L. (TsAr) (*right*) and a 250 year-old rachis with several empty spikelets of *T. aestivum* L. (TaIt) (*left*) from two historical buildings in Switzerland were used for DNA extraction

DNA extraction

Spikelets of *T. spelta* L. from Arlesheim (TsAr) were extracted 35 times (10–15 spikelets per sample) in seven extraction series, including five series consisting only of TsAr. The rachis of *T. aestivum* from Itingen (TaIt) was ground and one aliquot each was used in three separate extraction series. Fresh leaves (70 mg) of TsOb were used for extraction.

DNA was extracted using a CTAB-based method (Schlumbaum et al. 1998), modified by grinding the samples in liquid nitrogen with quartz sand, adding 5% 2-mercaptoethanol to the extraction buffer, and overnight precipitation of DNA at 4°C. One extraction series (No. 7) was performed using the Nucleon PhytoPure kit (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. The extracts were further cleaned with a silica extraction method (Höss and Pääbo 1993) in cases where the presence of PCR-inhibitors was indicated.

PCR, cloning and sequencing

PCR was carried out in 50-µl reactions using a Progene thermal cycler (Techne, Cambrigde, UK) or a PTC-100 (MJ-Research, Watertown, Mass.). Two regions of nuclear DNA were PCR-amplified: an approximately 240-bp fragment of the high-molecular-weight (HMW) glutenin promoter region (HGP) from the six homoeologous genes in hexaploid wheat and the internal transcribed spacer 2 (ITS2) region of the ribosomal DNA (rDNA). Glutenin primers and PCR conditions used to amplify HGP were described previously (Schlumbaum et al. 1998). ITS2 amplification was carried out to authenticate amplification of HGP in historical wheat. Primers and PCR conditions (universal primers rD4 and rD5) amplifying a 300-bp fragment from the 5.8S rDNA into the 26S rDNA were described by Deakin et al. (1998). Wheat-specific primers PITS3 5′-ACGCCAAAACACGCTCCCAAC-3′ and PITS4 5′-CTGGGGTCGCGGTCGAAG-3′ were designed based on a se-

quence alignment of the rDNA region from *T. aestivum* (EMBL acc. Z11761), *Triticum speltoides* Tausch. (EMBL acc. Z11762), *Triticum monococcum* (EMBL acc. L11581) and other gramineae (Hsiao et al. 1995). They amplify a fragment of approximately 235 bp. The PCR conditions were the same as for the 300-bp fragment (Deakin et al. 1998).

Re-amplifications were done using 1 µl from the first reaction in an additional 20 cycles, with identical conditions as before.

A CTAB extract of *Triticum boeoticum* Boiss. emend. Schiem. (Genomes A^bA^b) was used as a positive control. PCR products were fractionated in 3% NuSieve agarose (FMC BioProducts, Rockland, Me.) and stained with ethidium bromide.

PCR products were cloned with the p-GEM-T Vector Kit (Promega, Madison, Wis.) according to the manufacturer's instructions. Plasmids were purified with a QIAGEN miniprep plasmid kit (QIAGEN, Hilden, D) and sequenced on a ABI Prism 310 Genetic Analyser (Perkin Elmer, Foster City, Calif.). Sequences were submitted to EMBL/Genebank with accession numbers AJ399880–AJ399898.

Data analysis

The ancient sequences were corrected for PCR errors according to "Authenticity criteria" and consensus sequences were created, if possible. All sequences from this study (excluding 41 bp of the primers) and 11 representative HGP sequences from the database of six different *Triticeae* species (EMBL acc. AJ131815; AJ132190; U39229; X03041; X03042; X98583-X98587; Y10966) were aligned using the Wisconsin package Version 10.0 (GCG, Madison, Wis.). Only one Glu-A1-2 TsAr sequence was detected in all extracts. A correction of putative PCR errors was therefore not possible.

For the phylogenetic analysis, the partial sequence Glu-A1-2 TaIt was completed by the corresponding segment of Glu-A1-2 from *T. aestivum* cv Galahad. The deletion of a basepair triplet at positions 105–107 was considered as one singular event and substituted for one transversion. Neighbour-joining (NJ) trees were calculated using ClustalW version 1.6 (Thompson et al. 1994) with parameters set for 1000 bootstrap replicates, the exclusion of positions with gaps and no correction for multiple substitutions. Pairwise genetic distances were calculated using Kimura's two-parameter model (transition/transversion ratio of 2.0) in the Dnadist programme of PHYLIP 3.57 (Felsenstein 1989) including two additional sequences from *T. monococcum* L. (EMBL acc. Y12401; Y12402).

Authenticity criteria

False positives are a common threat to molecular studies of ancient specimens, where only minute quantities of DNA are available (Handt et al. 1994). Therefore criteria have to be applied to authenticate the ancient origin of the analysed DNA (Cooper and Poinar 2000).

Historical wheat samples were processed in physically separated laboratories designated exclusively for extracting ancient DNA, and dedicated material was used, for example separate pipettes, sterile ART pipette tips with filters (Molecular BioProducts, Inc. San Diego, Calif.) and disposable plasticware. Nondisposables such as pestle and mortar were bleached and autoclaved. Equipment and solutions were irradiated in a UV Stratalinker 2400 (Stratagene, La Jolla, Calif.) at maximum energy for at least 15 min. Two extraction blanks were included in every extraction series. The ITS2 region was amplified as a control region to confirm the presence of authentic DNA.

Identical sequences, identified in independent extractions of a historical sample were considered authentic. Variations occurring only in one of several extracts were considered as PCR errors. A variation unknown in the identified HGP gene but common in another HGP gene was considered as a site of jumping PCR, i.e. the polymerase created a hybrid sequence during the PCR by 'jumping' between templates of different HGP genes (Pääbo et al. 1990).

Results

PCR products from the historical wheat samples

Amplification of the HGP fragment yielded a product of the expected 240 bp in 10 out of 35 extracts (28.6%) from *T. spelta* 'Arlesheim' (TsAr) and in two out of three extracts (66%) from *T. aestivum* 'Itingen' (TaIt) (Table 1). From the ITS2 region a product of the expected size was amplified with the universal primers in 9 extracts out of 29 from TsAr (31%) and with the wheat specific primers in two extracts out of three from TaIt (66%) (Table 1). The amplification of both the HGP and the ITS2 region was successful in one extract from TsAr (1f) and in two extracts from TaIt (6c, 7e).

Sequence analysis of the HMW glutenin promoter region

Eight of the ten PCR products of the HGP from TsAr were cloned and 48 clones were sequenced. Of these, 35 sequences obtained from six PCR products (Table 2a: 1d, 1f, 3a, 4d, 4e and 4h) were considered authentic according to criteria listed in Methods. All six HMW glutenin genes (HMW Glu-A1-1, A1-2, B1-1, B1-2, D1-1 and D1-2) were identified with high similarity (>96%) to the corresponding published genes.

Table 1 PCR amplifications of a 240-bp fragment of the promoter region of HMW glutenin subunit gene (HGP) and a 300/235-bp fragment of the ITS2 region from DNA extracts of desiccated historical *T. spelta* (TsAr) and *T. aestivum* (TaIt)

Extraction series	No. of extracts from TsAr	No. of PCR products:		No. of extracts from TaIt	No. of PCR products:	
		HGP	ITS2		HGP	ITS2
1 a–f	6	2[a]	4	–	–	–
2 a–d	4	0	n.d.[b]	–	–	–
3 a–i	9	2[a]	4[a]	–	–	–
4 a–h	8	3	0	–	–	–
5 a, b	2	1	1	–	–	–
6 a–c	2	0	n.d.	1	1	1
7 a–e	4	2	0	1	1	1
8 a	–[c]	–	–	1	0	0
Total	35	10	9	3	2	2

[a] After reamplification
[b] 'n.d.'=not determined
[c] '–' no extract included in this series

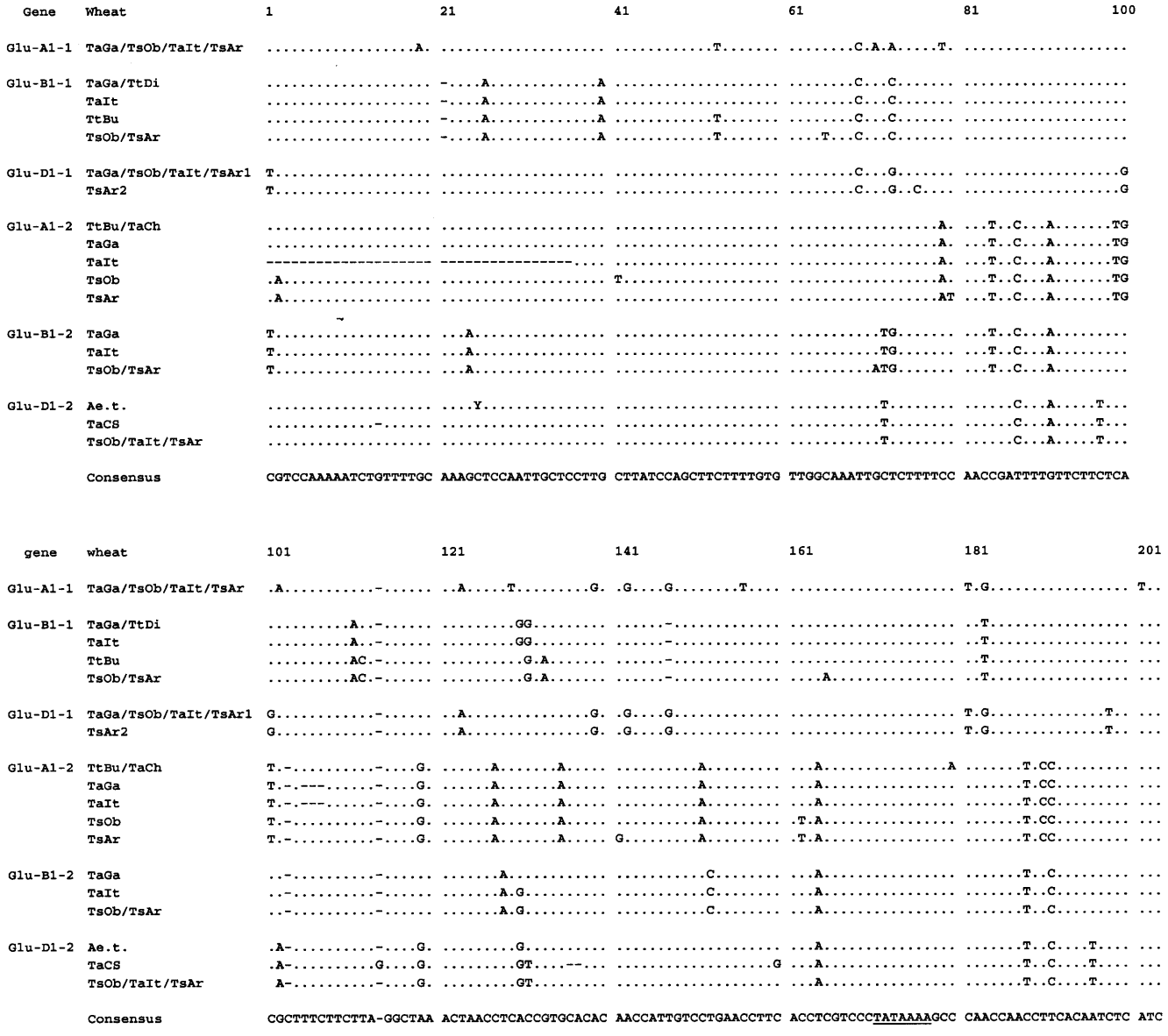

Fig. 2 Sequence alignment of the partial HMW glutenin promoter region including 19 sequences found in this study and 11 sequences of di-, tetra- and hexa-ploid wheat from the EMBL/Genebank database. PCR primers are excluded, 200 bp are shown, *numbering* starts at the first base of the consensus sequence after the forward primer, the TATA box is *underlined*. The sources (including abbreviations) of the aligned sequences were: *T. spelta* 'Arlesheim' (TsAr), *T. aestivum* 'Itingen' (TaIt), *T. spelta* 'Oberkulmer' (TsOb), *T. aestivum* cv Galahad (TaGa), *T. aestivum* cv Cheyenne (TaCh), *T. aestivum* cv Chinese Spring (TaCS), *T. turgidum* var. *dinurum* (TtDi), *T. turgidum* var. *buccale* (TtBu) and *Ae. tauschii* (Ae.t.)

The sequences obtained from extract 3h did not align to any published sequence in the database. Sequences obtained from one cloned PCR product of TsAr from series 7 were highly similar to corresponding wheat genes but were excluded from further analysis because several mutations, occurring in all five genes detected, did not allow unambigous reconstruction of the original sequences.

Twenty four clones of both PCR products of the sample TaIt were sequenced. All six HMW glutenin genes were identified (Table 2b). The number of genes detected per PCR product was variable in TsAr and TaIt (Table 2).

Sequence comparison

Sequences were aligned and differences are discussed compared to the most similar allele each. The alignment of the HGP sequences from TsOb, TsAr and TaIt with representative sequences from the database showed that the genes Glu-A1-2, Glu-B1-1 and Glu-B1-2 from the two spelt samples TsOb and TsAr differed from all other *Triticeae* (Fig. 2).

The Glu-A1-2 allele from TsOb varied from the most-similar sequence of *T. aestivum* cv Cheyenne at four positions (A for a G at pos. 2, T for a C at pos. 41, T for a C at pos. 162 and C for an A at pos. 180). The single

Table 2 Number of clones corresponding to the genes of the HWM glutenin promoter region (HGP) obtained from PCR products from extracts of historical *T. spelta* Arlesheim (**a**) and *T. aestivum* Itingen (**b**). The notation of the extracts refers to Table 1

a

Extracts from TsAr	Number of HGP alleles obtained from the extract						No. of HGP genes detected
	Glu-A1–1	Glu-A1–2	Glu-B1–1	Glu-B1–2	Glu-D1–1	Glu-D1–2	
1d	–	–	–	–	–	4	1
1f	–	–	–	–	9	–	1
3a	–	–	–	5	–	–	1
4d	1	–	2	1	1	6	5
4e	–	–	1	–	–	1	2
4h	–	1	1	–	2	–	3

b

Extracts from TaIt	Number of HPG alleles obtained from the extract						No. of HGP genes detected
	Glu-A1–1	Glu-A1–2	Glu-B1–1	Glu-B1–2	Glu-D1–1	Glu-D1–2	
6c	4	2	2	2	2	6	6
7e	1	–	1	–	3	1	4

Glu-A1-2 sequence obtained from TsAr shared the variations at pos. 2, 162 and 180 with TsOb, but not at pos. 41. This was also found in Glu-A1-2 sequences from several modern spelt accessions (unpublished results). The sequence from TsAr also varied from the allele of *T. aestivum* cv Cheyenne at positions 80 and 141 (T for C and G for A, respectively). These differences are so far unknown from modern sequences and might also be PCR errors. The partial allele of Glu-A1-2 from TaIt was identical to the allele from *T. aestivum* cv Galahad, characterized by a missing triplet at position 105–107, in contrast to wheats with the triplet, such as *T. aestivum* cv Cheyenne (Fig. 2).

Various polymorphic alleles of the Glu-B1-1 gene are known. The Glu-B1-1 alleles found in the two hexaploid spelt samples TsOb and TsAr were identical, but they differed at two positions (T for C at pos. 65 and A for C at pos. 165) to the most similar allele of *Triticum turgidum* var. *buccale*, with which they share four differences (T for C at pos. 53, A for G at pos. 130 and 133 and C for T at pos. 112) to the corresponding allele of *T. aestivum* cv Galahad and *T. turgidum* var. *dinurum*. The allele of Glu-B1-1 from TaIt was identical to the allele from *T. aestivum* cv Galahad.

The Glu-B1-2 allele of TsOb and TsAr varied from the allele of *T. aestivum* cv Galahad by an A for G at pos. 71 and a G for an A at pos. 130. The allele of TaIt is identical to Glu-B1-2 of *T. aestivum* cv Galahad except for a G at pos. 130, shared with TsOb and TsAr.

Identical alleles of the genes Glu-A1-1, Glu-D1-1 and Glu-D1-2 were found in both spelt wheats TsOb and TsAr and the bread wheat TaIt. The Glu-A1-1 and Glu-D1-1 alleles were also identical to the corresponding alleles published for tetra- and hexaploid wheat so far. In TsAr one additional allele of Glu-D1-1 (TsAr2) was identified in two independant extracts (1f, 4d) with a C to T transition at pos. 76 compared to the other allele Glu-D1-1TsAr1 from TsAr, identified in the extracts 1f and 4h. The allele of Glu-D1-2 in TsOb, TsAr and TaIt varied from the most-similar corresponding allele of *Triticum tauschii* by a T instead of C at position 131, also found in *T. aestivum* cv Chinese Spring (Fig. 2).

None of the mutations described here affected regulatory promoter units such as the TATA box (pos. 171–174) or the CCAT sequence (pos. 143–146) (Reddy and Appels 1993). The PCR error rate of the *Taq* polymerase was calculated for TsAr: $1.61*10^{-3}$, TaIt: $1.46*10^{-3}$, TsOb: $1.72*10^{-3}$errors per base pair. Observed events of jumping PCR were in TsAr: 0.06, TaIt: 0.29, TsOb: 0.19 events of jumping PCR per HGP sequence.

Sequence analysis of the ITS2 region

From 38 clones of two PCR products amplified from the sample TsAr with the universal ITS2 primers, approximately 75% of the obtained ITS2 sequences corresponded to wheat; 25% of the ITS2 sequences originated from other plants. Their presence was confirmed in a morphological survey on the weeds associated with the sample (Blatter et al., in preparation) and strengthened the evidence that authentic DNA was obtained from the historical specimen.

Phylogenetic analysis

To explore the relationship between the HGP of spelt and other wheats, the sequences obtained in this study and 11 previously published sequences of tetra- and hexa-ploid wheat and *Ae. tauschii* were used for a neighbour-joining (NJ) analysis based on the calculation of percentage similarities (Fig. 3). Pairwise genetic distances were calculated using Kimura's two-parameter model (Table 3).

Table 3 Pairwise genetic distances from 19 sequences of 200 bp of the HMW glutenin promoter region were calculated using Kimura's two-parameter model. Abbreviations are as in Fig. 2 (in addition: Tmon=*T. monococcum*)

Glu-	A1–2 TtBu	A1–2 TaGa	A1–2 TsOb	A1–2 TsAr	B1–2 TaGa	B1–2 TaIt	B1–2 TsOb	D1–2 Ae.t.	D1–2 TaCS	D1–2 TsOb	A1–1 TaGa	A1–1 Tmon	B1–1 TaGa	B1–1 TtBu	B1–1 TsOb	D1–1 TaGa	D1–1 TsAr2
A1–2Tmon	0.036	0.037	0.046	0.052	0.086	0.091	0.097	0.080	0.087	0.085	0.176	0.170	0.129	0.141	0.153	0.144	0.151
A1–2TtBu	–	0.010	0.020	0.025	0.085	0.091	0.096	0.079	0.092	0.084	0.174	0.168	0.133	0.145	0.158	0.137	0.143
A1–2TaGa		–	0.021	0.026	0.086	0.092	0.098	0.080	0.093	0.086	0.177	0.171	0.136	0.148	0.161	0.140	0.146
A1–2TsOb			–	0.015	0.096	0.101	0.107	0.090	0.103	0.095	0.186	0.174	0.145	0.157	0.170	0.149	0.155
A1–2TsAr				–	0.101	0.107	0.113	0.095	0.103	0.101	0.192	0.180	0.151	0.163	0.176	0.155	0.161
B1–2TaGa					–	0.005	0.010	0.057	0.069	0.062	0.148	0.142	0.103	0.115	0.127	0.101	0.107
B1–2TaIt						–	0.005	0.052	0.063	0.057	0.153	0.148	0.097	0.121	0.133	0.107	0.112
B1–2TsOb							–	0.057	0.069	0.062	0.148	0.142	0.103	0.126	0.139	0.112	0.118
D1–2Ae.t								–	0.010	0.005	0.136	0.143	0.091	0.114	0.126	0.118	0.124
D1–2TaCS									–	0.005	0.151	0.157	0.099	0.122	0.135	0.132	0.138
D1–2TsOb										–	0.142	0.148	0.091	0.114	0.126	0.124	0.130
A1–1TaGa											–	0.030	0.103	0.103	0.115	0.062	0.067
A1–1Tmon												–	0.097	0.097	0.109	0.057	0.062
B1–1TaGa													–	0.020	0.031	0.074	0.080
B1–1TtBu														–	0.010	0.085	0.091
B1–1TsOb															–	0.097	0.103
T1–1TaGa																–	0.005

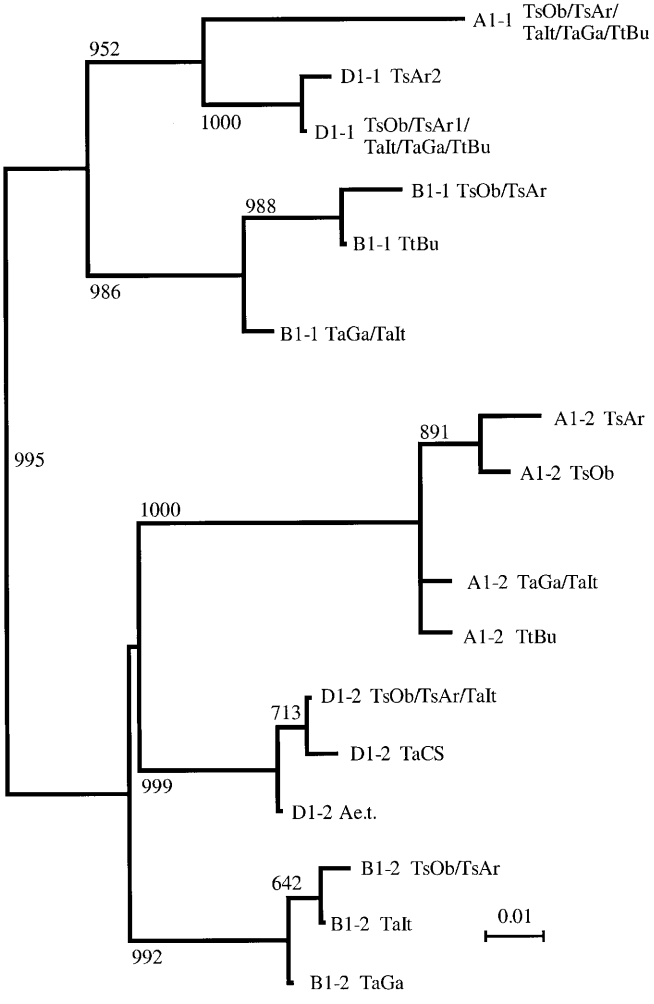

Fig. 3 Unrooted neighbour-joining tree of 200 bp of the HMW glutenin promoter region constructed using the sequences from the alignment in Fig. 2. Bootstrap values of 1000 replicate trees are shown. The *bar* refers to 0.01% sequence variation. Abbreviations are as in Fig. 2

The NJ-tree was divided into two main branches formed by the orthologous Glu-1-1 and Glu-1-2 genes (Fig. 3). Each of the six glutenin genes in hexaploid wheat clearly formed a separate clade. Most variation within the HGP was observed in the Glu-B1-1 and Glu-A1-2 clades. With a genetic distance of 0.020 the Glu-B1-1 clade was split into two distinct subclades, described previously by Brown (1999) and named alpha and beta. A high number of bootstrap replicas supported the stability of the split. The allele of the two spelt samples TsOb and TsAr clustered within the so-called beta subclade together with some tetraploid wheats, represented by *T. turgidum* L. var. *buccale* (Fig. 3). The minimal genetic distance from the spelt Glu-B1-1 allele to a HGP sequence from bread wheat was 0.031. Similar genetic distances separated the Glu-A1-1 and Glu-A1-2 alleles from *T. monococcum* (genomes A^bA^b) to the corresponding alleles of the A^uA^u genome, for example, in bread wheat (0.030 and 0.036, respectively; Table 3). The allele of Glu-B1-1 from TaIt clustered within the alpha subclade together with the known alleles of all other *T. aestivum* and with tetraploid wheat, such as *T. turgidum* var. *dinurum*.

The Glu-A1-2 clade was equally split into two subclades. One lineage is formed exclusively by the two spelt sequences TsOb and TsAr (0.020 and 0.025 compared to bread wheat, respectively; Table 3), the second subclade by all other wheats, including TaIt (Fig. 3).

Little variation was discovered within alleles of HGP Glu-B1-2 and D1-2. Whereas the Glu-D1-2 alleles from the spelt samples are identical to the allele of *T. aestivum* from Itingen, the Glu-B1-2 alleles from spelt differ from the alleles of bread wheat and tetraploid wheat by at least one base pair (≥0.005).

The clades of the genes Glu-A1-1 and Glu-D1-1 showed no variation in the analysed HGP sequence, except for a second allele of Glu-D1-1 from TsAr, which varied by one mutation. An NJ-tree including all

60 available HMW sequences from the EMBL/Genebank database confirmed the topology, branch lengths and clade stability of the NJ-tree in Fig. 3, which includes only representative sequences (data not shown).

Discussion

The repeated extraction of ancient DNA from the two wheat specimens *T. spelta* Arlesheim (TsAr) and *T. aestivum* Itingen (TaIt), and the successful amplification of a fragment of the promoter from all the expected six HMW glutenin single-copy genes, shows the value of desiccated historical plant remains for molecular analysis. Based on allelic variations each historical specimen could be clearly assigned to its corresponding taxonomic wheat unit. This confirmed that, despite its short length, the 240-bp fragment of the HGP was sufficiently informative to infer wheat phylogeny.

Evidence for polyphyletic alleles in spelt and bread wheat

The alleles of the HGP from the 300 year-old spelt TsAr were identical or highly similar to alleles of recent *T. spelta* Oberkulmer (TsOb), whereas the alleles from the 250 year-old bread wheat TaIt corresponded best to alleles from modern *T. aestivum* (e.g. cv Galahad). Spelt and bread wheat are considered closely related (Sears 1974; Zohary and Hopf 2000). However, the Glu-A1-2, Glu-B1-1, and to a lesser extent also the Glu-B1-2, alleles in the analysed spelt differed from the bread wheat alleles published so far. The genetic distance between the spelt Glu-A1-2 and Glu-B1-1 alleles and the most similar alleles in bread wheat was at least 0.020–0.025 and 0.031, respectively. Interestingly, similar genetic distances were found for each of the Glu-A1-1 and Glu-A1-2 HGP alleles (0.030 and 0.036, respectively) of *T. monococcum* compared to the most similar alleles of polyploid wheats such as *T. dicoccum* and *T. aestivum*. Whereas *T. monococcum* (genome A^bA^b) is the domesticate of wild einkorn (*Triticum boeoticum* Boiss.), the A-genome of polyploid wheats is closely related to the wild wheat *Triticum urartu* Tuman. (genome A^uA^u) (Dvorák et al. 1992).

With an estimated mutation rate for synonymous substitutions in nuclear monocotyledon genes of 5.1–7.1$\times 10^{-9}$ substitutions site^{-1} year^{-1} (Wolfe et al. 1989) the number of base changes between the spelt and bread wheat alleles (4–5 in Glu-A1-2 and 6 in Glu-B1-1) are too many to have evolved since the emergence of hexaploid wheat approximately 7000–8000 years ago (Zohary and Hopf 2000).

These considerations suggest a polyphyletic origin of the Glu-A1-2 and B1-1 alleles present in the analysed spelt and bread wheat samples.

Scenarios of a polyphyletic origin of spelt and bread wheat alleles

Two main scenarios can explain a polyphyletic origin of alleles on the A- and B-genome. Spelt and bread wheat could have descended from different hexaploid hybrids of tetraploid wheat and *Ae. tauschii*, i.e. the different glutenin alleles represent the genetic variation in the ancestral tetraploid wheat. The second possibility is an introgression of tetraploid wheat into hexaploid wheat (e.g., Schiemann 1951; Liu and Tsunewaki 1991).

The Glu-D1-1 and Glu-D1-2 sequences in the analysed spelt and bread wheat were each identical, but the Glu-D1-2 allele varied from corresponding published sequences, which indicates that several *Ae. tauschii* were involved in the evolution of hexaploid wheat. This is in agreement with a previous study, where RFLP markers on the D-genome provided strong evidence that hexaploid wheat evolved several times by the hybridization of *Ae. tauschii* and tetraploid wheat, and that subsequently a common genepool of the D-genome of hexaploid wheat was formed by hybridisation, recombination, selection and genetic drift (Dvorák et al. 1998a). The reported differentiation between spelt and bread wheat was very limited compared to the difference to the most-related subpopulation of *Ae. tauschii*, which suggests that differentiation in hexaploid wheat is not due to a different ancestral *Ae. tauschii* (Dvorák et al. 1998a; von Büren 2001).

Similar to the D-genome, the evolution of a common genepool of the A- and B-genomes in hexaploid wheat would be expected if no introgression of tetraploid into hexaploid wheat had occurred. An introgression would increase the variation and differentiation in the A- and B-genomes among hexaploid wheat without altering the D-genome. Both a high variation of the A- and B-genomes and a differentiation of spelt and bread wheat was indicated by the different Glu-A1-2, B1-1 and B1-2 alleles in the analysed spelt and bread wheat. A higher genetic variation of the A- and B-genomes compared to the D-genome (Liu and Tsunewaki 1991; Siedler et al. 1994), and a differentiation of spelt from bread wheat, was also found in previous studies with various modern wheat samples (Liu and Tsunewaki 1991; Siedler et al. 1994; von Büren et al. 2000; Bertin et al. 2001; von Büren 2001). These observations support that our results are not the effect of a random variation because of the small number of samples analysed, but more-likely reflect a differentiation among hexaploid wheats.

Evidence for a secondary origin of European spelt by a hybridisation of tetraploid and hexaploid wheat is the Glu-B1-1 beta subclade, which was formed by the different alleles from spelt and some tetraploid wheats, whereas all bread wheats clustered in the alpha subclade. Brown (1999) reported that in 262 cultivated tetraploid wheats the alpha alleles were common and widely distributed, whereas the beta alleles were found in a minority of the tetraploid wheats. If the Glu-B1-1 beta alleles were restricted initially to tetraploid wheat, this suggests

that the Glu-B1-1 allele in the spelt TsAr and TsOb originates from a tetraploid wheat and the D-genome from a hexaploid wheat. A similar result was found with the gamma gliadin alleles on the B-genome that are present in spelt and some tetraploid wheats but not found in bread wheat (von Büren 2001). Since only a limited number of individual hexaploid wheats would have contributed to the D-genome of the new speltoid alloploids, a bottleneck effect could explain the limited differentiation among the D-genomes.

The result of our analysis of the HGP region of spelt and bread wheat can be explained by European spelt originating from a hybridization of tetraploid and hexaploid wheat. This scenario may be reflected in the different history of emmer and bread wheat compared to spelt, as suggested by the archaeological evidence (Nesbitt and Samuel 1996). However, our conclusions have to be considered as preliminary, since the examined specimens represent an exceptional, but limited, collection of different hexaploid wheats from a very restricted area spanning a time period of at least 250 years. Nevertheless, the analysis of historical specimens is important for investigating a broad genetic range of spelt, especially considering the genetic narrowness of modern spelt cultivars. For example, *T. spelta* 'Oberkulmer' is the parent of many accessions analysed in studies on modern spelt (Siedler et al. 1994; von Büren et al. 2000; Bertin et al. 2001). To obtain conclusive evidence on how and where European spelt evolved, it will be necessary to analyse additional recent and historical spelt accessions from different geographical regions and to include other genetic markers. The presence of the polyphyletic alleles in the historical samples shows that the observed differences are at least 250 years old, but it can not tell us whether this is the result of events that happened in the Bronze Age, when the first large quantities of spelt are found in the archaeological record in Europe, or in a later, e.g. Medieval, time period. More information will depend on the availability of wheat specimens of the time periods in question, from which authentic DNA can be extracted.

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References

Allaby RG, Jones MK, Brown TA (1994) DNA in charred wheat grains from the Iron Age hillford at Danebury, England. Antiquity 68:126-132

Allaby RG, Banerjee M, Brown TA (1999) Evolution of the high-molecular-weight glutenin loci of the A, B, D and G-genomes of wheat. Genome 42:296–307

Bertin P, Grégoire D, Massart S, Froidmont Dd (2001) Genetic diversity among European cultivated spelt revealed by microsatellites. Theor Appl Genet 102:148–156

Bertsch F (1943) Der Dinkel. Landw Jahrbuch 92:241–252

Brown TA (1999) How ancient DNA may help in understanding the origin and spread of agriculture. Proc R Soc Lond B Biol Sci 354:89–98

Büren M von (2001) Lüthy J, Hübner P (2000) A spelt specific g-gliadin gene: discovery and detection. Theor Appl Genet 100:271–279

Büren M von (2001) Polymorphism in two homeologous gamma-gliadin genes and the evolution of cultivated wheat. Genet Res Crop Evol 48:205–220

Cooper A, Poinar HN (2000) Ancient DNA: do it right or not at all. Science 28:1139

Deakin WJ, Rowley-Conwy P, Shaw CH (1998) The *Sorghum* of Qasr Ibrim: reconstructing DNA templates from ancient seeds. Ancient Biomolec 2:117–124

Dvorák J, Terlizzi Pd, Zhang H-B, Resta P (1992) The evolution of polyploid wheats: identification of the A-genome donor species. Genome 36:21–31

Dvorák J, Luo M-C, Yang Z-L, Zhang H-B (1998a) The structure of the *Aegilops tauschii* genepool and the evolution of hexaploid wheat. Theor Appl Genet 97:657–670

Dvorák J, Luo M-C, Yang Z-L (1998b) Genetic evidence on the original of *Triticum aestivum* L. In: Damania AB, Valkoun J, Willcox G, Qualset CO (eds) The origins of agriculture and crop domestication. The Harlan Symposium. ICARDA, Aleppo, pp 235–251

Felsenstein J (1989) PHYLIP – phylogene inference package (version 3.2). Cladistics 5:164–166

Flaksberger C (1930) Ursprungszentrum und geographische Verbreitung des Spelzes (*Triticum spelta* L.). Angewandte Botanik 12:86–99

Handt O, Höss M, Krings M, Pääbo S (1994) Ancient DNA: methodological challenges. Experientia 50:524–529

Hillman GC, Mason S, deMoulins D, Nesbitt M (1996) Identification of archaeological remains of wheat: the 1992 London workshop. Circaea 12:195–209

Höss M, Pääbo S (1993) DNA extraction from Pleistocene bones by silica-based purification method. Nucleic Acids Res 21: 3913–3914

Hsiao C, Chatterton NJ, Asay KH, Jensen KB (I995) Phylogenetic relationships of the monogenomic species of the wheat tribe, Triticae (Poaceae), inferred from nuclear rDNA (internal transcribed spacer) sequences. Genome 38:211–223

Jacomet S, Brombacher C, Dick M (1989) Archäobotanik am Zürichsee. Orell Füssli, Zürich

Jacomet S, Kreuz A (1999) Lehrbuch der Archäobotanik. Ulmer, Stuttgart

Kukuck H, Schiemann E (1957) Über das Vorkommen von Spelz und Emmer (*Triticum spelta* and *T. dicoccum* (Schübl.) im Iran. Z Pflanzenzücht 38:383–396

Liu YG, Tsunewaki K (1991) Restriction fragment length polymorphism (RFLP) analysis in wheat. II. Linkage maps of the RFLP sites in common wheat. Jpn J Genet 5:617–633

Luo MC, Yang ZL, Dvorak J (2000) The Q locus of Iranian and European spelt wheat. Theor Appl Genet 100:602–606

Lupton FGH (1987) Wheat breeding: its scientific basis. Chapman and Hall, London

MacKey J (1996) Species relationship in *Triticum*. Hereditas 2:237–276

Maier U (1996) Morphological studies of free-threshing wheat ears from a Neolithic site in southwest Germany, and the history of the naked wheats. Veg Hist Archaeobot 5:39–55

McFadden ES, Sears ER (1946) The origin of *Triticum spelta* and its free-thresing hexaploid relatives. J Hered 37:81–89

Nesbitt M, Samuel D (1996) From staple crop to extinction? The archeology and history of the hulled wheats. In: Padulosi S, Hammer K, Heller J, Pacoli C (eds) Hulled wheats. International Plant Genetic Resources Institute, Rome, Itay, pp 41–100

O'Donoghue K, Clampham A, Evershed RP, Brown TA (1996) Remarkable preservation of biomolecules in ancient radish seeds. Proc R Soc Lond B Biol Sci 263:541–547

Pääbo S, Irwin DM, Wilson AC (1990) DNA damage promotes jumping between templates during enzymatic amplification. J Biol Chem 265:4718–4721

Payne PI (1987) Genetics of wheat storage proteins in the effect of allelic variation on bread-making quality. Annu Rev Plant Phys Plant Mol Biol 38:141–153

Percival J (1974) The wheat plant. Duckworth, London

Reddy P, Appels R (1993) Analysis of a genomic DNA segment carrying the wheat high-molecular-weight (HMW) glutenin Bx17 subunit and its use as an RFLP marker. Theor Appl Genet 85:616–624

Rollo F, LaMarca A, Amici A (1987) Nucleic acids in mummified plant seeds: screening of 2 specimens by gel-electrophoresis, molecular hybridization and DNA cloning. Theor Appl Genet 73:501–505

Savolainen V, Cuénoud P, Spichiger R, Martinez MDP, Chrèvecoeur M, Manen J-F (1995). The use of herbarium specimens in DNA phylogenetics: evaluation and improvement. Plant Syst Evol 197:87–98

Schiemann E (1951) New results on the history of cultivated cereals. J Heredity 5:305–320

Schlumbaum A, Neuhaus J-M, Jacomet S (1998) Coexistence of tetraploid and hexaploid naked wheat in a Neolithic lake dwelling of Central Europe. Evidence from morphology and ancient DNA. J Arch Sci 25:1111–1118

Sears ER (1974) The wheats and their relatives. In. King RC (ed) Handbook of genetics, vol 2/5: plants, plant viruses and protists. Plenum Press, New York, pp 59–91

Siedler H, Messmer MM, Schachermayr GM, Winzeler H, Keller B (1994) Genetic diversity in European wheat and spelt breeding material based on RFLP data. Theor Appl Genet 88:994–1003

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Wolfe K, Sharp PM, Li W-H (1989) Rates of synonymous substitution in plant nuclear genes. J Mol Evol 29:208–211

Zohary D, Hopf M (2000) Domestication of plants in the world. Oxford University Press, Oxford, pp 19–59