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High-resolution organellar genome analysis of *Triticum* and *Aegilops* sheds new light on cytoplasm evolution in wheat

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Abstract We have utilised polymorphic chloroplast microsatellites to analyse cytoplasmic relationships between accessions in the genera *Triticum* and *Aegilops*. Sequencing of PCR products revealed point mutations and insertions/deletions in addition to the standard repeat length expansion/contraction which most likely represent ancient synapomorphies. Phylogenetic analyses revealed three distinct groups of accessions. One of these contained all the non-*Aegilops speltoides* S-type cytoplasm species, another comprised almost exclusively A, C, D, M, N, T and U cytoplasm-type accessions and the third contained the polyploid *Triticum* species and all the *Ae. speltoides* accessions, further confirming that *Ae. speltoides* or a closely related but now extinct species was the original B-genome donor of cultivated polyploid wheat. Successive decreases in levels of genetic diversity due to domestication were also observed. Finally, we highlight the importance of elucidating longer-term evolutionary processes operating at microsatellite repeat loci.

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Introduction

The genus *Triticum*, which includes bread wheat (*T. aestivum*), represents one of the world's most important cereal crops and exists as a polyploid series of diploid, tetraploid and hexaploid species complexes. The evolution of polyploid wheats has been the subject of much debate in recent years and is known to involve the related genus *Aegilops*. Whilst it is now generally accepted that the diploid donors of the A and D genomes were *T. urartu* and *T. tauschii* (synonym *Ae. squarrosa*) respectively (Kihara 1924; McFadden and Sears 1946), the identity of the donor(s) of the B and G genomes remains a contentious issue. Many different species have been proposed as the original donor of these genomes but it is now largely believed that the progenitor was a member of the *Sitopsis* section of the genus *Aegilops* and of these, either *Ae. bicornis*, *Ae. longissima* or, most likely, *Ae. speltoides* (Tsunewaki and Ogihara 1983).

It is known that the original B-genome donor was the maternal parent of Emmer wheat ($2n=4\times=28$, genome designation AABB) and that the Emmer group (*T. dicoccum*, *T. dicoccoides* and *T. durum*) included the maternal parent of *T. aestivum* (AABBDD) since the cytoplasm of both Emmer wheat and common wheat is that associated with the B-genome. Consequently, molecular genetic analysis of the cytoplasmic (chloroplast and mitochondrial) genomes has been employed to elucidate the identity of the original maternal donor (Graur et al. 1989; Terachi et al. 1990; Terachi and Tsunewaki 1992; Miyashita et al. 1994; Mori et al. 1997; Wang et al. 1997). The apparently contradictory findings of many of these studies suggests that intraspecific variation in either the donor or in *T. aestivum* may have been responsible for the lack of correlation between cytoplasms and the subsequent inability to unequivocally assign a donor to the B genome. This may be due to the fact that within the potential progenitor species, an individual containing the donor cytoplasm genotype was not studied or that the technique used was of insufficiently high resolution to detect any intraspecific varia-

Table 1 *Triticum* and *Aegilops* species used in this study (by cytoplasm type). Numbers in parentheses in "Accession" column represent multiple individuals within a species which share a common cytoplasm haplotype

Species	Code	Accession	Ploidy	Cytoplasm	Haplotype
T. monococcum	MON	All (31)	2×	A	X
T. urartu	URA	DVG2	2×	A	XII
		18913			VIII
		110649			VII
		110794			VII
		110795			VIII
		110809			VIII
		110810			VIII
		500253			X
		500518			X
		600084			X
		1010006			X
Ae. speltoides	SPE	(22)	2×	B	XV
		400079			XVIII
		400955			XIX
		401847			XVIII
		401931			XVI
		403029			XVIII
		403085			XIX
T. dicoccoides	DES	1060007	4×	B	XV
T. dicoccum	DUM	All (3)	4×	B	XV
T. durum	DUR	(8)	4×	B	XVIII
		Kyperounda			XV
		Sabil			XV
T. aestivum	AES	(34)	6×	B	XV
		Bouquet			XVII
		Capelle-Desprez			XVII
		600071			IX
Ae. caudata	CAU	400441	2×	C	V
		2090003			III
T. tauschii	TAU	Torfrida	2×	D	XI
		G4483			X
		18902			VI
		110730			VI
		110825			VI
		110856			VI
		500301			X
		500319			X
		500340			X
		600026			IX
		699933			X
Ae. cylindrica	CYL	2100003	4×	D	I
Ae. ventricosa	VEN	600052	4×	D	IX
		2270001			XI
Ae. crassa	CRA	110672	6×	D^2	X
		2250001	6×	D^2	VI
T. araraticum	ARA	1150002	4×	G	XVIII
Ae. comosa	COM	600075	2×	M	X
		2110002	2×		XIV
Ae. uniaristata	UNI	2120001	2×	N	XIII
Ae. bicornis	BIC	2190001	2×	S^b	II
Ae. sharonensis	SHA	2170001	2×	S^l	II
Ae. variabilis	VAR	2070003	2×	S^v	II
Ae. mutica	MUT	G4535	2×	T	IX
		2130005			III
Ae. umbellulata	UMB	2010036	2×	U	III
Ae. biuncialis	BIU	2060001	4×	U	III
Ae. triuncialis	TRU	110785	4×	U	VI
		2080001	4×		IV
Ae. triaristata	TRA	2030002	4×	U	III
		2040001	6×		III
Ae. columnaris	COL	2050002	4×	U^2	III

tion present. Recently, a new class of cytoplasmic markers, chloroplast microsatellites (or simple sequence repeats: cpSSRs), has been developed which allows the high-resolution analysis of chloroplast genomes (for reviews see Powell et al. 1996; Provan et al. 2001). We have previously used such markers to study many aspects of crop plant evolution such as documenting multiple events in the domestication of maize from its wild progenitor (Provan et al. 1999a) and the existence of cytoplasmic bottlenecks in the domestication of cultivated barley (Provan et al. 1999b) and in the modern European cultivated potato (Provan et al. 1999c).

There has been much debate over the use of microsatellite markers to reconstruct phylogenies (for reviews see Jarne and Lagoda 1996; Goldstein and Pollock 1997; Provan et al. 2001). The main perceived problems include uncertainty over mutational models operating at microsatellite loci, homoplasy (the generation of alleles that are identical in state but which do not share the same evolutionary history) and possible constraints on allele sizes, which will result in a non-linear relationship between differences in allele size and evolutionary time. Furthermore, since microsatellites are generally assayed as differences in PCR product lengths using gel electrophoresis, the true nature of mutation in microsatellite analysis is usually unclear. It is now becoming apparent that sequencing microsatellite products can provide valuable insights into the evolutionary history of these loci. Although microsatellites represent useful markers below the species level or between closely related species, as the divergence time between the taxa under study increases, so does the likelihood that simple stepwise mutation models cannot accurately account for all the variation (observed and unobserved) between those taxa.

In this study we have used polymorphic cpSSRs developed from wheat chloroplast sequences to analyse cytoplasmic relationships between accessions in the genera *Triticum* and *Aegilops*. We have used a combination of standard simple sequence length polymorphism (SSLP) analysis and sequencing of SSLP-PCR products to resolve relationships at different taxonomic levels.

Materials and methods

Plant material

Seeds were obtained from S. M. Reader and J. W. Snape (John Innes Centre, UK) and were grown in petri dishes. A list of the species used is given in Table 1. Total genomic DNA of most of the *T. aestivum* and *Ae. tauschii* lines and all *Ae. cylindrica* lines was isolated according to a procedure described in Dvorak et al. (1998). The remaining genomic DNAs were isolated as follows. Leaf tissue was ground to a fine powder with a chilled mortar and pestle in the presence of glass beads and liquid nitrogen. In a 15 ml polypropylene conical tube, 3 ml of Plant DNAzol (Life Technologies) for every gram of fresh tissue was added and mixed by shaking. An equal volume of phenol chloroform was added, vortexed briefly, and centrifuged for 7 min at 3,000 rpm. The upper phase was transferred to a new 15 ml conical polypropylene tube. DNA was precipitated with 0.7 vol. cold isopropanol, removed with a Pasteur pipette hook and placed in a clean Eppendorf tube with 1 ml of 70% ethanol. The sample was centrifuged for 10 min at 3,000 rpm and the ethanol was decanted. The pellet was dried of all residual ethanol and resuspended in 300 μl TE with 2 μl RNAse (10 mg/ml).

Primer design and polymerase chain reaction

Partial wheat chloroplast sequences in the EMBL database were searched for all mononucleotide repeats of eight bases or more using the STRINGSEARCH and FINDPATTERNS programs (Genetics Computer Group). Primers were designed to amplify mononucleotide repeats in non-coding regions using the program PRIMER (v0.5) and are given in Table 2. PCR was carried out in a total volume of 10 μl containing 50 ng genomic DNA, 10 pmol ^{32}P end-labelled forward primer, 10 pmol reverse primer, 1× PCR reaction buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1% Triton X-100), 15 mM $MgCl_2$, 0.05 U *Taq* polymerase (Promega). Reactions were carried out on a Perkin Elmer 9600 thermal cycler using the following parameters: initial denaturation at 94ºC for 3 min; 30 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, extension at 72ºC for 30 s; final extension at 72ºC for 5 min. After addition of 10 μl loading buffer (95% formamide), products were resolved on 6% denaturing polyacrylamide gels containing 1× TBE buffer and 8 M urea at 80 W constant power for 2 h. Gels were transferred onto 3MM blotting paper (Whatman) and exposed to X-ray film overnight at –70ºC. PCR products were also sequenced using an ABI 377 automated sequencer and standard fluorescent dye terminator chemistry.

Data analysis

Synapomorphies were used to construct a "framework" tree showing broad-scale relationships between groups of individuals. Within these individual groups, genetic distances between individuals were calculated based on the absolute size difference of alleles (D_{AD}) between cpSSR haplotypes using the computer program MICROSAT (v1.5, Eric Minch, Stanford University, USA). This distance is similar to Goldstein's $\delta\mu^2$ (Goldstein et al. 1995) and is

Table 2 Primers used in this study

Locus	Repeat	Location	Primers (5′-3′)	T_m (°C)	Size (bp)
*atp*1	$(A)_9$	Downstream of *atp*H	GGACTTAGTTGTGGCACTAGCG GAATTGCGGAAGCAAATACC	60	126
*cfo*3	$(A)_{12}$	*cfo*I intron	TGCCCTTTTTTAACCAATGC CATGGTCAGCAAAGTTGTTTC	60	187
*rps*2	$(A)_9$	Upstream of *rp*S2	CGTAATGGAATGGTAGG AGCCGTTCTAGCGAG	50	197
*trn*T1	$(T)_{11}$	Upstream of *trn*T	GTTCCAGAATCCGCCTAT TCGATATTTTGAGAGCTCAA	55	126

appropriate for closely related species. Within-species diversity values based on D_{AD} distances were calculated by averaging all pairwise distances between individuals within each species. Neighbour-joining trees were constructed using the NEIGHBOR and DRAWGRAM options in the PHYLIP package (v3.57c, Felsenstein 1995).

Results

Mutational processes at chloroplast microsatellite loci in wheat

Sequencing of the microsatellite-containing PCR products revealed mutations other than the standard expansion/contraction of the mononucleotide repeat at two of the four loci (*atp*1 and *cfo*3; see Table 3). At the *atp*1 locus, four of the accessions (2100003, 2070003, 2170001 and 2190001) were characterised by an A→C substitution in the mononucleotide repeat (haplotypes I and II). With the exception of *Triticum aestivum* accession 600071, all the polyploid *Triticum* species and all the *Aegilops speltoides* accessions were characterised by a "standard" $(A)_n$ repeat at the *cfo*3 locus (haplotypes XV–XX). In the remaining accessions studied, this repeat was interrupted by another substitution and, in addition, these accessions also contained a 2 bp insertion without the microsatellite region (haplotypes III–XIV). In all, a total of 20 different haplotypes was detected (Table 3).

Evolution of wild and cultivated wheat species

The point mutations and insertions/deletions described above are relatively rare compared to the bi-directional mutational processes operating at microsatellite loci and, as a result, they probably represent more ancient mutations than the microsatellite length variation observed. Consequently, they were used as synapomorphies to construct a "framework" tree delineating the three main groups of accessions (Fig. 1a) with each subtree constructed from the microsatellite-based D_{AD} distance metric (Fig. 1b–d). The first group, containing both the *atp*1 and *cfo*3 synapomorphies, comprised four accessions sharing two very closely related haplotypes (I and II). Three of these were the S-type cytoplasm accessions included in the study. The second, most diverse group contained the *cfo*3 synapomorphy only and included the A, C, D, M, N, T and U cytoplasm accessions with the

Table 3 Chloroplast SSR haplotypes detected in this study. Haplotypes I–XIV also contained a 2 bp insertion outside the repeat region at the *cfo*3 locus

Haplotype	Locus			
	*atp*1	*rps*2	*trn*T	*cfo*3[a]
I	$(A)_5C(A)_3$	$(A)_8$	$(T)_9$	$(A)_{10}T(A)_6$
II	$(A)_5C(A)_3$	$(A)_8$	$(T)_9$	$(A)_{11}T(A)_6$
III	$(A)_9$	$(A)_8$	$(T)_9$	$(A)_9T(A)_6$
IV	$(A)_9$	$(A)_8$	$(T)_9$	$(A)_{10}T(A)_6$
V	$(A)_9$	$(A)_{10}$	$(T)_9$	$(A)_9T(A)_6$
VI	$(A)_9$	$(A)_{10}$	$(T)_9$	$(A)_{10}T(A)_6$
VII	$(A)_9$	$(A)_{10}$	$(T)_{10}$	$(A)_{10}T(A)_6$
VIII	$(A)_9$	$(A)_{10}$	$(T)_{10}$	$(A)_{11}T(A)_6$
IX	$(A)_9$	$(A)_8$	$(T)_9$	$(A)_9T(A)_6$
X	$(A)_{10}$	$(A)_8$	$(T)_9$	$(A)_{10}T(A)_6$
XI	$(A)_{10}$	$(A)_9$	$(T)_9$	$(A)_{10}T(A)_6$
XII	$(A)_{10}$	$(A)_8$	$(T)_9$	$(A)_{11}T(A)_6$
XIII	$(A)_{10}$	$(A)_8$	$(T)_{10}$	$(A)_{10}T(A)_6$
XIV	$(A)_{10}$	$(A)_8$	$(T)_{10}$	$(A)_{11}T(A)_6$
XV	$(A)_9$	$(A)_9$	$(T)_{11}$	$(A)_{11}$
XVI	$(A)_9$	$(A)_9$	$(T)_{11}$	$(A)_{14}$
XVII	$(A)_9$	$(A)_9$	$(T)_{11}$	$(A)_{12}$
XVIII	$(A)_9$	$(A)_9$	$(T)_{10}$	$(A)_{11}$
XIX	$(A)_9$	$(A)_9$	$(T)_{10}$	$(A)_{12}$
XX	$(A)_9$	$(A)_9$	$(T)_9$	$(A)_{11}$

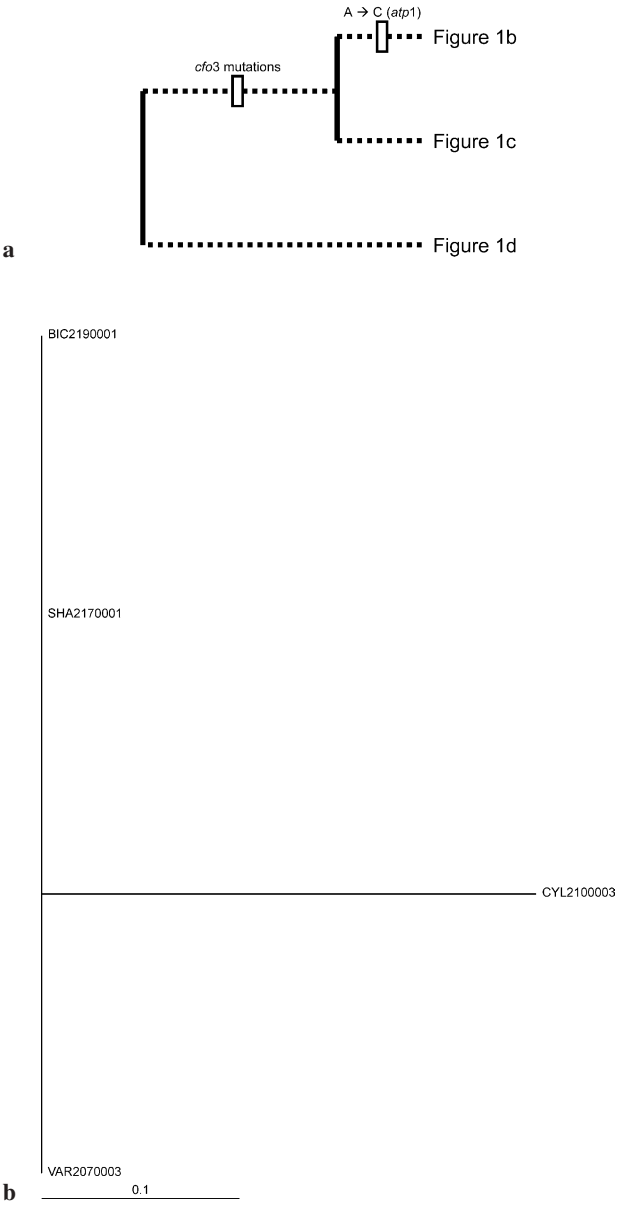
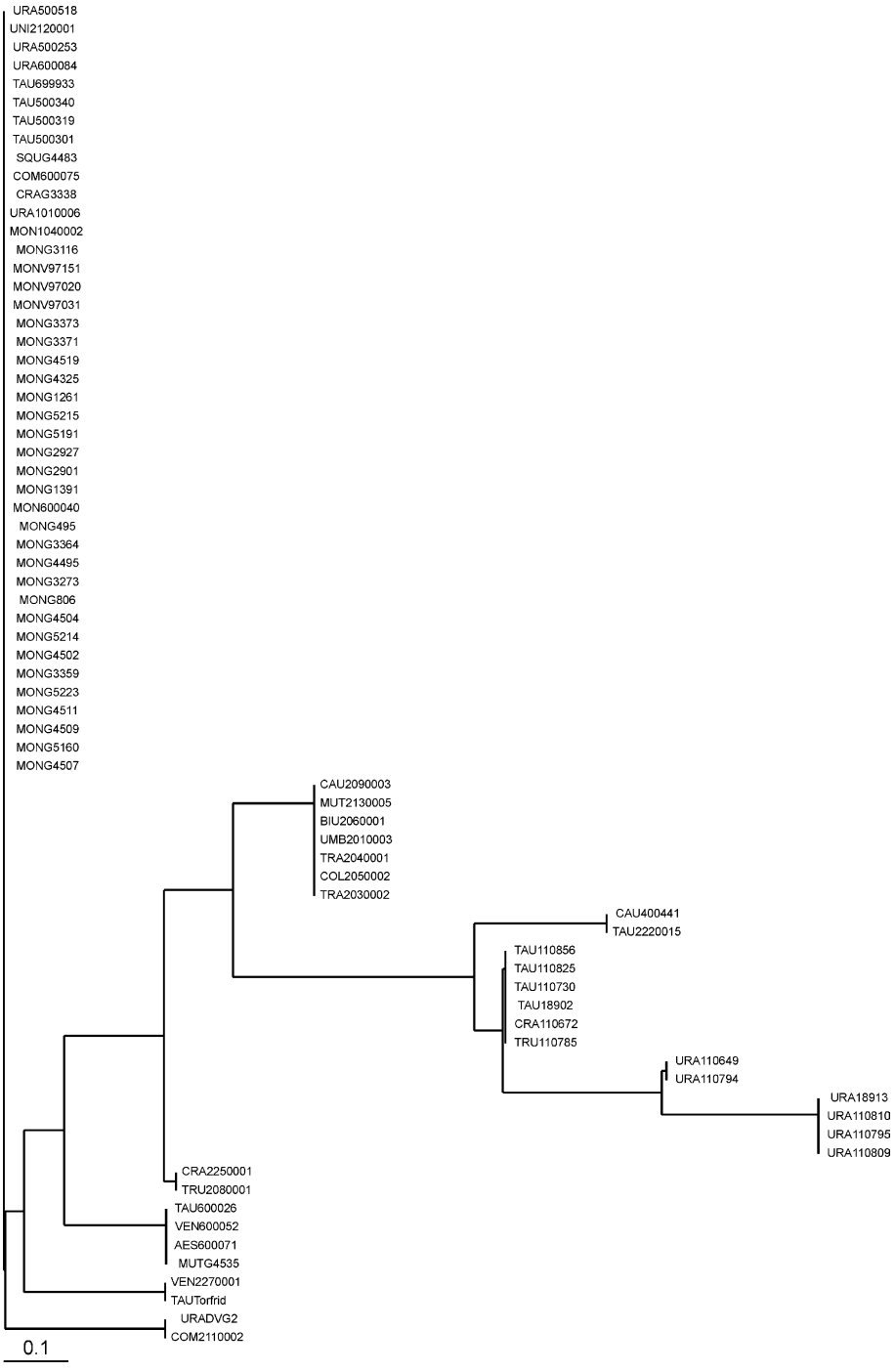


Fig. 1a–d Phylogenetic trees showing relationships between accessions analysed in this study. **a** The "framework" tree. **b–d** The subtrees derived from the framework tree

Fig. 1c



exception of *Ae. cylindrica* 2100003 (C-type cytoplasm). *T. aestivum* 600071 was also included in this group. The final group comprised all polyploid *Triticum* species (B-type cytoplasm, with the exception of *T. araraticum* which possesses the related G-type cytoplasm) and all the *Ae. speltoides* accessions.

D_{AD}-based diversity values were calculated for A-genome and B-genome wild and cultivated species to determine the effects of domestication on cytoplasmic diversity. The wild A-genome diploid *T. urartu* had a diversity value of 0.682, whereas the cultivated diploid *T. monococcum* was completely monomorphic. In the species with the B-type cytoplasm, *Ae. speltoides* had a diversity value of 0.190 whereas the cultivated polyploids *T. dicoccum*, *T. durum* and *T. aestivum* exhibited diversity values of 0.000, 0.089 and 0.027 respectively (Table 4).

Discussion

New molecular genetic techniques have facilitated the detailed examination of patterns of diversity and evolu-

Fig. 1d

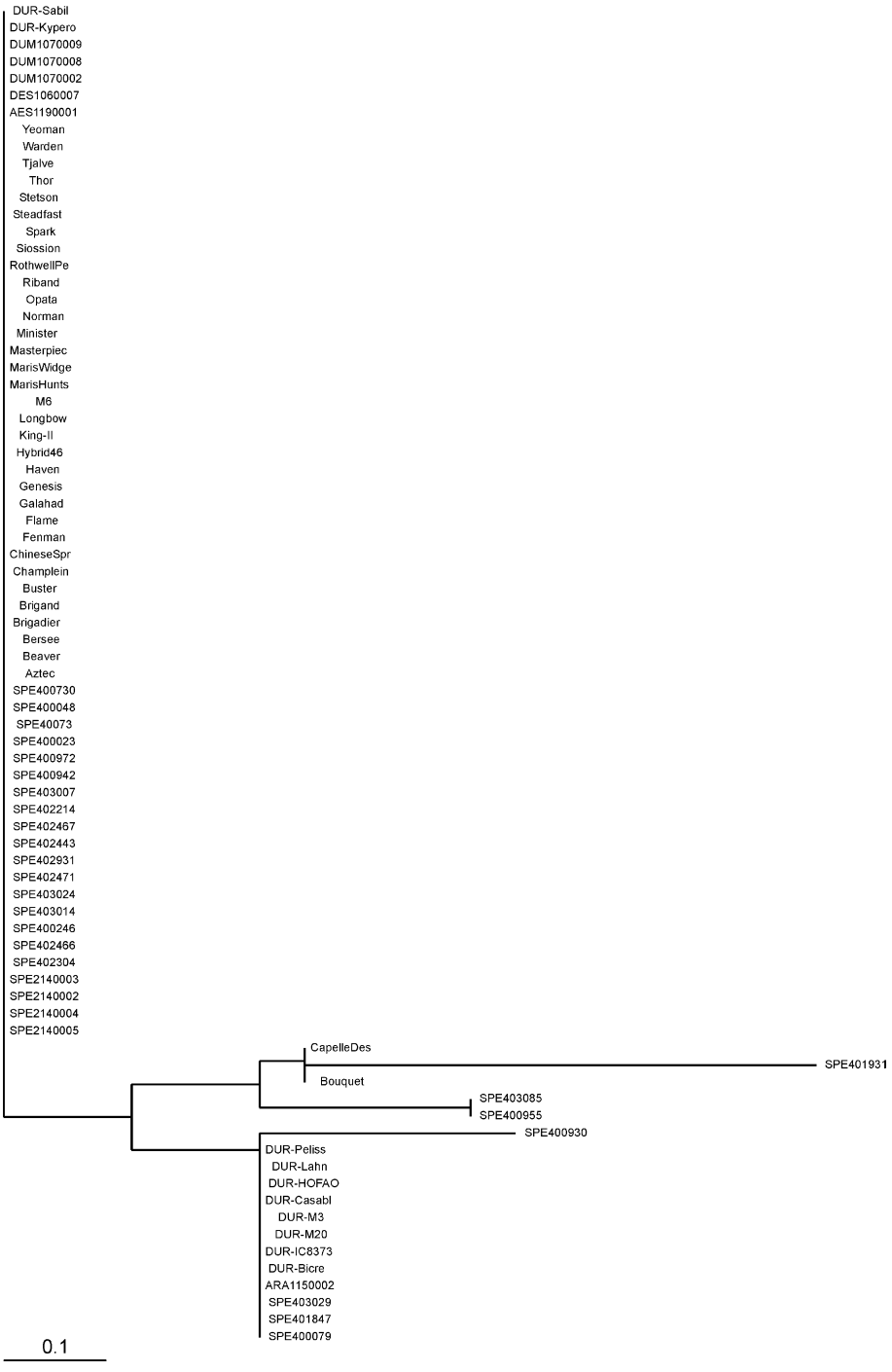


Table 4 D_{AD} diversity values in wild and domesticated wheats

Genome	Wild		Domesticated					
	2×		2×		4×		6×	
A	*T. urartu* (*n*=12)	0.682	*T. monococcum* (*n*=31)	0.000	–		–	
B	*Ae. speltoides* (*n*=28)	0.190	–	–	*T. durum* (*n*=10)	0.089	*T. aestivum* (*n*=36)	0.027
	–	–	–	–	*T. dicoccum* (*n*=3)	0.000	–	–

tion in many crop species. In a recent study using amplified fragment length polymorphism (AFLP) analysis, Heun et al. identified the Karačadac mountains of southeast Turkey as the likely site of domestication of einkorn wheat (Heun et al. 1997). The development of chloroplast SSRs in *Triticum* and *Aegilops* provides a new, high-resolution technique for the cytoplasmic analysis of wheat and its wild relatives. Using cpSSRs we have detected variation both within species and within cytoplasm types which had previously gone largely undetected in studies utilising RFLPs, a feature also highlighted in a separate cpSSR study in wheat by Ishii et al. (2001). This is consistent with our earlier cpSSR studies in other cereals where we detected higher levels of variation than earlier RFLP studies in rice (Provan et al. 1996, 1997), maize (Provan et al. 1999a) and barley (Provan et al. 1999b). The assessment of such intraspecific variation is a crucial factor in elucidating evolutionary histories, as will be discussed later.

It is now evident that mutational processes other than simple bi-directional length changes occur at microsatellite loci (Jarne and Lagoda 1996). Sequencing of the four microsatellite loci used in the present study revealed both point mutations and an insertion/deletion mutation. Since these are much rarer than changes in microsatellite length, particularly in the chloroplast genome (Provan et al. 1999d), it is likely that they represent ancient synapomorphies. These synapomorphies were found to significantly delineate groups of accessions, particularly with respect to the polyploid cultivated wheats. This extra information revealed by sequencing the cpSSR loci has provided further insights into the evolution of wheat species that would not be apparent from SSLP-PCR alone.

Cytoplasmic relationships within *Triticum* and *Aegilops*

Although there have been doubts raised over the applicability of cpSSRs to phylogenetic reconstruction due to perceived high levels of homoplasy resulting from the bidirectional mutational processes operating at tandem repeat loci (Doyle et al. 1998), it would seem that unless extended evolutionary time spans are considered, homoplasy may not be such a problem, particularly within a genus or closely related genera. This should be even less of a problem when using chloroplast microsatellite loci since the associated mutation rates are lower than those found at their nuclear counterparts (Provan et al. 1999d). Previous studies using cpSSRs in cereals have managed to reconstruct accurately evolutionary relationships below the family level in rice (Provan et al. 1997), maize (Provan et al. 1999a) and barley (Provan et al. 1999b), as well as in potato (Bryan et al. 1999). Indeed, the lack of a mutually exclusive discrimination between *Triticum* and *Aegilops* is consistent with many previous suggestions that all wheat species should be treated as congeneric (McFadden and Sears 1946; Bowden 1956). The haplotypes exhibited by the diploid *Triticum* accessions, *T. urartu* and *T. monococcum* (both A cytoplasm) were very different from those found in the polyploid B and G cytoplasm *Triticum* species. The grouping of the diploid wheats with *Aegilops* species rather than the other member of the genus *Triticum* is consistent with previous chloroplast studies using RFLP (Ogihara and Tsunewaki 1988) and PCR-SSCP (Wang et al. 1997) as well as the mitochondrial RFLP work of Graur et al. (1989) and Mori et al. (1997). It is interesting to note that the A-cytoplasm of *T. urartu* displayed considerably more variation (six haplotypes in 13 accessions; D_{AD} diversity value of 0.682) than the related A^2 cytoplasm of *T. monococcum*, which displayed a uniform cytoplasm. A similar uniformity was also evident in the U cytoplasm species studied, which is entirely in agreement with the findings of Wang et al. (1997). Likewise, we noticed the close relationship between the U- and T-type cytoplasms also highlighted by Wang et al. (1997).

With the exception of *Ae. speltoides*, the *Aegilops* species which share variants of the S-type cytoplasm (*Ae. bicornis* [S^b], *Ae. sharonensis* [S^l] and *Ae. variabilis* [S^v]) share a single haplotype. The close relationship between these cytoplasm types and the large difference in cytoplasm types between *Ae. speltoides* and the rest of the S-cytoplasm species has been well documented using various techniques (Ogihara and Tsunewaki 1988; Graur et al. 1989; Wang et al. 1997). All the *Ae. speltoides* accessions in this study grouped with the polyploid B cytoplasm *Triticum* species, with the exception of *T. aestivum* 600071, and *T. araraticum*, which contains the closely related G cytoplasm.

The position of *Ae. cylindrica* accession CYL210003 is obviously erroneous, since it is grouped with the S-subtype cytoplasm accessions rather than with the other U-cytoplasm accessions. This is most likely due to a recent spontaneous mutation at the *atp*I locus, with a resulting $(A)_9 \rightarrow (A)_5C(A)_3$ interruption of the mononucleotide repeat. Without this single mutation, the *Ae. cylindrica* accession would exhibit haplotype IV, which is found in a group in the phylogram which comprises mostly U- and D-cytoplasm species.

Evolution of cultivated polyploid wheat

Previous studies utilising molecular markers in crop plants have highlighted a progressive loss of diversity between cultivated species and their wild progenitors (Tanksley and McCouch 1997). A comparison of cultivated diploid and polyploid wheats with their purported ancestral species confirms this fact. *T. monococcum*, a cultivated A-genome diploid, was completely monomorphic whereas its progenitor *T. urartu* exhibited a D_{AD} diversity value of 0.682. Likewise, when tracing the evolution/domestication of the B-type cytoplasm (believed to be *Ae. speltoides* [2×]→*T. durum* [4×]→*T. aestivum* [6×]), there is a progressive loss of diversity from 0.190 to 0.089 to 0.027. A similar scenario was found in a study on wild and cultivated barley using cpSSRs, where there was a progressive decrease in

diversity from wild barley (*Hordeum spontaneum*), through domesticated landraces of *H. vulgare* to cultivars (Provan et al. 1999b).

Although it is now widely believed that *Ae. speltoides* or a closely related (and possibly now extinct) relative was the original B-genome donor of polyploid wheats, many recent studies have presented evidence which apparently contradicts this hypothesis. Mori et al. (1997), working on PCR-RFLP of the *cox*II mitochondrial region and Randhawa et al. (1997), who used SDS-PAGE analysis of HMW glutenin, both agreed that while *Ae. speltoides* probably donated the G-genome to Timopheevi wheat, their results suggested that it was not the B-genome donor of Emmer wheat. Likewise, Ogihara and Tsunewaki (1988) could find no diploid species that shared a cytoplasm type with Emmer wheat. In all these cases, the level of resolution of the techniques used was probably too low to effectively detect intraspecific polymorphism, and thus the concept of different genotypes existing within a species, one or more of which could have been the B-genome donor, could not be addressed. This is exacerbated when a single accession or very few accessions are taken as being representative of a species. The PCR-SSCP technique used by Wang et al. (1997) has the potential to reveal more variation than RFLPs but did not manage to identify any diploid species which shared a cytotype with Emmer wheat. Our work suggests that *Ae. speltoides* may well have been the B-genome donor. Furthermore, the results presented here highlight the importance of considering and assessing levels of intraspecific variation and suggest that the analysis of variation at chloroplast tandem repeat loci provides an ideal, high-resolution tool for such studies. In addition to standard SSLP analysis, sequencing of these regions has also revealed information on other mutations (substitutions and indels) which shed further light on the longer-term processes in the evolution of wheat cytoplasm.

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