Theor Appl Genet (2000) 100:221–231

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Evolutionary features of chondriome divergence in *Triticum* (wheat) and *Aegilops* shown by RFLP analysis of mitochondrial DNAs

Received: 20 March 1999 / Accepted: 22 June 1999

Abstract The first comprehensive analysis was made of restriction fragment length polymorphism (RFLP) of the mitochondrial (mt) DNA of two related genera, *Triticum* (wheat) and *Aegilops*. This led to clarification of the nature of mtDNA variability and the inference of the phylogeny of the mitochondrial genomes (=chondriome). Forty-six alloplasmic lines and one euplasmic line of common wheat (2n = 42, genomes AABBDD) carrying plasmons (cytoplasmic genomes) of 47 accessions belonging to 33 species were used. This consisted of nearly all the *Triticum* and *Aegilops* species. RFLP analysis, carried out with seven mitochondrial gene probes (7.0 kb in total) in combination with three restriction endonucleases, found marked variation: Of the 168 bands detected, 165 were variable (98.2%), indicative that there is extremely high mtDNA variability in these genera. This high variability is attributed to the variation present in the intergenic regions. Most of the variation was between chondriomes of different plasmon types; only 8 bands (4.8%) between those of the same plasmon types were variable, evidence of clear chondriome divergence between different plasmon types. The first comprehensive phylogenetic trees of the chondriome were constructed on the basis of genetic distances. All but 1 of the polyploids had chondriomes closely related to those of 1 putative parent, indicative of uniparental chondriome transmission at the time of polyploid formation. The chondriome showed parallel evolutionary divergence to the plastome (chloroplast genome). Use of a minimum set of 3 mtDNA probe-enzyme combinations is proposed for tentative plasmon type identification and the screening of new plasmon types in those genera.

Communicated by J.W. Snape

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Key words Mitochondrial DNA · RFLP analysis · *Triticum* · *Aegilops* · Plasmon phylogeny

Introduction

The terms plasmon, chondriome and plastome are used to indicate the cytoplasmic, mitochondrial (mt) and chloroplast (cp) genomes, respectively (Rieger et al. 1991). The term genome refers only to the nuclear genome.

Two related genera, *Triticum* and *Aegilops*, comprise more than 30 species that include many tetra- and hexaploids. The interspecific relationships between these species have been studied extensively using genome analysis (Lilienfeld 1951; Kimber 1983) and plasmon analysis (Tsunewaki 1996). To date, more than 500 alloplasmic lines of common wheats carrying plasmons of other *Triticum* and *Aegilops* species have been produced (Tsunewaki et al. 1996). These alloplasmic wheats have various novel phenotypes produced by the alien plasmons; parthenogenesis, delayed growth, pistillody and premature seed germination, as well as the commonly observed male sterility (Tsunewaki 1996 and references therein). The alloplasmic common wheats have also been used to analyze molecular variation in the organellar genomes because of their efficient production of seeds with alien plasmons when fertile alloplasmic lines are used (Terachi and Tsunewaki 1986; Ogihara and Tsunewaki 1988). The differentiation of at least 22 types of plasmon has been recognized among *Triticum* and *Aegilops* species based on phenotypic effects and the molecular variation of organellar genomes (Tsunewaki 1996).

Analysis of molecular variation in the organellar genomes of *Triticum* and *Aegilops* was initiated by Vedel et al. (1978). The chondriomes of these species appear to carry heterogeneous DNA molecules (Terachi and Tsunewaki 1992), which has also been reported in other plants (Sederoff et al. 1981; Palmer and Herbon 1986; Small et al. 1987). Breiman (1987) and Terachi and Tsunewaki (1992) used Southern hybridization to study

restriction fragment length polymorphism (RFLP) of the mtDNAs of those genera. The latter researchers analyzed 19 allo- and euplasmic wheats, representing 17 plasmon types, and constructed the first phylogenetic tree of the chondriome. Recently, Wang et al. (1997) made polymerase chain reaction (PCR)-SSCP (single-strand conformational polymorphism) analyses of chloroplast and mitochondrial genes in almost the same materials as those used in the study reported here. They detected a number of variations in the chloroplast genes, but there was strikingly little variation in the mitochondrial genes, indicative that they were highly conserved during evolution.

We describe here RFLP analysis carried out with 18 probe-enzyme combinations of the chondriomes of *Triticum* and *Aegilops* using 46 alloplasmic lines and one euplasmic line of common wheat. These lines represent all 22 plasmon types known in these genera. Seven mtDNA

Table 1 Genetic constitution of the 47 alloplasmic lines and the euplasmic (control) line analyzed

Line	Plasmon type[a]	Nuclear genotype[b]	Plasmon donor				
			Group or section	Species, subspecies, variety or cultivar	Ploidy	Genome constitution (n)[c]	Code no.
btc	A	CS	Einkorn	*T. boeoticum* var *boeoticum*	2 x	A	01
mnc	A^2	CS	Einkorn	*T. monococcum* var *flavescens*	2 x	A	16
dcd	B	CS	Emmer	*T. dicoccodes* var *spontaneon.*	4 x	AB	21
dcm	B	CS	Emmer	*T. dicoccum* cv Vernal	4 x	AB	22
*ast*1	B	CS	Common	*T. aestivum* cv CS	6 x	ABD	52
*ast*2	B	CS	Common	*T. aestivum* cv Penjamo	6 x	ABD	11
*ast*3	B	CS	Common	*T. aestivum* ssp. *tibetanum*	6 x	ABD	58
*arr*1	G	Splt	Timopheevi	*T. araraticum*	4 x	AG	23
*arr*2	G	Splt	Timopheevi	*T. araraticum*	4 x	AG	24
tmp	G	Splt	Timopheevi	*T. timopheevi*	4 x	AG	25
zhk	G	Splt	Timopheevi	*T. zhukovskyi*	6 x	AAG	51
*mtc*1	T	CS	Amblyopyrum	*Ae. mutica*	2 x	T	13
*mtc*2	T^2	CS	Amblyopyrum	*Ae. mutica*	2 x	T	14
cms	M	CS	Comopyrum	*Ae. comosa*	2 x	M	05
hld	M^h	CS	Comopyrum	*Ae. heldreichii*	2 x	M	06
unr	N	CS	Comopyrum	*Ae. uniaristata*	2 x	N	07
*cdt*1	C	Cmp	Cylindropyrum	*Ae. caudata* var *polyathera*	2 x	C	02
*cdt*2	C	CS	Cylindropyrum	*Ae. caudata*	2 x	C	27
cyl	D	CS	Cylindropyrum	*Ae. cylindrica*	4 x	CD	28
umb	U	JF	Polyeides	*Ae. umbellulata*	2 x	U	03
*trn*1	U	CS	Polyeides	*Ae. triuncialis*	4 x	UC	26
*trn*2	C^2	Cmp	Polyeides	*Ae. triuncialis*	4 x	UC	38
*bnc*1	U	CS	Polyeides	*Ae. biuncialis*	4 x	U*M*	29
*bnc*2	U	CS	Polyeides	*Ae. biuncialis*	4 x	U*M*	37
clm	U^2	CS	Polyeides	*Ae. columnaris*	4 x	U*M*	30
ovt	M^o	CS	Polyeides	*Ae. ovata*	4 x	U*M*	31
*trr*1	U	Cmp	Polyeides	*Ae. triaristata*	4 x	U*M*	32
*trr*2	U	CS	Polyeides	*Ae. triaristata*	6 x	*UMN*	54
*trr*3	U	CS	Polyeides	*Ae. triaristata*	6 x	*UMN*	57
*kts*1	S^v	CS	Polyeides	*Ae. kotschyi*	4 x	U*S*	33
*kts*2	S^v	Cmp	Polyeides	*Ae. kotschyi*	4 x	U*S*	39
vrb	S^v	CS	Polyeides	*Ae. variabilis*	4 x	U*S*	34
*spl*1	S	CS	Sitopsis	*Ae. speltoides* var *ligustica*	2 x	S	08
*spl*2	G^2	Mch	Sitopsis	*Ae. speltoides* var *aucheri*	2 x	S	09
*spl*3	G	Splt	Sitopsis	*Ae. speltoides* var *ligustica*	2 x	S	15
*spl*4	S	CS	Sitopsis	*Ae. speltoides* var *aucheri*	2 x	S	17
bcr	S^b	CS	Sitopsis	*Ae. bicornis*	2 x	S^l	12
lng	S^{l2}	CS	Sitopsis	*Ae. longissima*	2 x	S^l	20
shr	S^l	CS	Sitopsis	*Ae. sharonensis*	2 x	S^l	10
srs	S^v	CS	Sitopsis	*Ae. searsii*	2 x	S^s	18
*sqr*1	D	CS	Vertebrata	*Ae. squarrosa*	2 x	D	04
*sqr*2	D	CS	Vertebrata	*Ae. squarrosa*	2 x	D	19
vnt	D	CS	Vertebrata	*Ae. ventricosa*	4 x	DN	36
*crs*1	D^2	CS	Vertebrata	*Ae. crassa*	4 x	D*M*	35
*crs*2	D^2	CS	Vertebrata	*Ae. crassa*	6 x	*DDM*	55
jvn	D^2	CS	Vertebrata	*Ae. juvenalis*	6 x	*DMN*	53
vvl	D^2	CS	Vertebrata	*Ae. vavilovii*	6 x	*DMS*	56

[a] The classification is based on phenotypic and molecular studies on wheat plasmons (Tsunewaki 1996)
[b] All common wheats: CS and JF are *T. aestivum* cv 'Chinese Spring' and cv 'Jones Fife', respectively, and Cmp, Splt and Mch are *T. compactum* cv 'No. 44', *T. spelta* var '*duhamelianum*' and *T. macha* var '*subletschchumicum*', respectively
[c] After Kimber and Tsunewaki (1988)

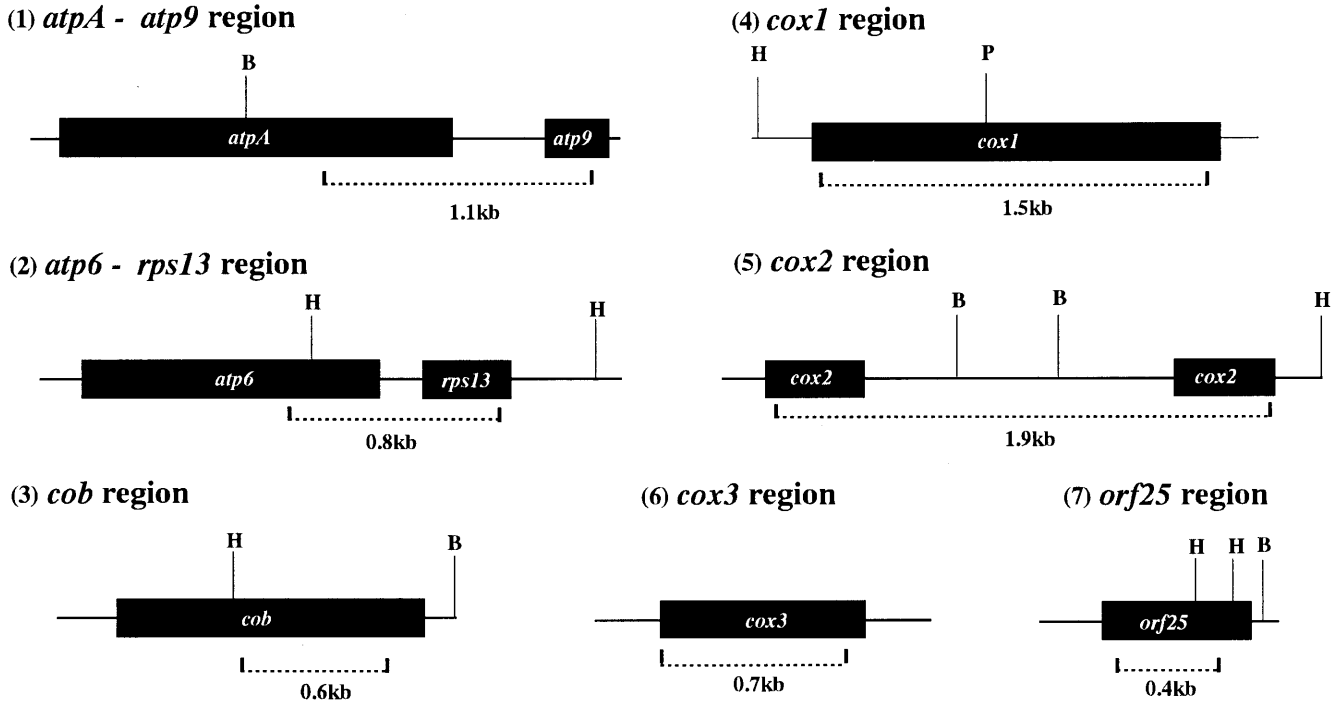

Fig. 1 Physical maps of the seven mitochondrial gene regions used as probes. *Solid box* Coding region(s) of each gene, *region* marked by a *dotted line* the mtDNA segment amplified by PCR – its size is given *below the line*, *B*, *H* and *P* respective restriction sites of *Bam*HI, *Hin*dIII and *Pst*I

Table 2 Primers used for PCR amplification of seven mitochondrial gene regions

MtDNA region	Primer sequence	
	Sense	Antisense
atpA-atp9	CAATGTGATCTCCATTACAGAG	AATAGCTTCGGTGAGAGCAAAG
atp6-rps13	GCACCTTTTTTAGTACTCCTTG	TCCATCTTGATGACGAATTCCA
cob	CATACCAGTAGTAGGAGATACC	CTCCACAGGTTGACATCC
cox1	CTAACCACAAGGATATTGGGAC	AGTTCTCCAAAAGTATGAAAGGC
cox2	TGATGCTGCGGAACCATGGCAA	GAGGATTAATTGATTGGATACCC
cox3	ATGATTGAATCTCAGAGGCATTC	TGCTTCAAAGCCAACGTGATGC
orf25	TCTATTTGTGCATCAAGTCCGAAG	TATATCGTCCTGAAGACGGATGCG

regions (totally 7.0 kb) were used as probes in combination with three 6-base cutters. Some characteristic features of mitochondrial genome evolution were identified in the two genera, and high mtDNA variation was useful for screening plasmon types.

Materials and methods

Plant material

Forty-six alloplasmic lines and one euplasmic line of common wheat that carry all the known plasmon types in *Triticum* and *Aegilops* were used (Table 1). These plasmons were introduced from 33 species that represent all the known genome types in these genera. Of the 47 lines, 37 had the nucleus of *T. aestivum* cv ′Chinese Spring', and the other 10 had the nucleus of *T. aestivum* cv 'Jones Fife', *T. compactum* cv 'No. 44', *T. spelta* var '*duhamelianum*' or *T. macha* var '*subletschchumicum*', all nuclear donors belonging to common wheat. Each alloplasmic line was backcrossed with its nucleus donor more than four times before use.

Total DNA extraction and mtDNA probe preparation

Total DNA was extracted from each line according to Liu et al. (1990). Seven mtDNA regions were amplified by PCR (Fig. 1) and used as probes. They included nine structural genes encoding the ATP synthase α and 9 subunits (*atpA* and *atp9*), the ATP synthase subunit 6 and ribosomal protein S13 (*atp6* and *rps13*), the apocytochrome b (*cob*), the cytochrome oxidase subunits, I, II and III (*cox1*, *cox2* and *cox3*) and ORF25 (*orf25*). References for these genes are given elsewhere (Wang et al. 1997). PCR was carried out using total DNA of *T. aestivum* cv 'Chinese Spring' as the template. Its primers, designed using published nucleotide sequences, were provided by T. Terachi, Kyoto Sangyo University, Japan (Table 2). A description of the PCR conditions is available upon request. The PCR products purified by column-chromatography were cloned with plasmid vectors. The authenticity of the cloned PCR fragments was checked by partial sequencing.

RFLP analysis

Approximately 0.5–0.75 μg of total DNA was digested with a 6-base-recognizing restriction enzyme, *Bam*HI, *Hin*dIII or *Pst*I, as

specified by the manufacturer (Takara Shuzo). The digested samples were separated on a 0.85% agarose gel and transferred to a Hybond N+ membrane (Amersham-Pharmacia). Probe labeling and hybridization were done with a DIG labeling and detection kit (Roche). The membranes were washed twice with 2 × SSC plus 0.1% SDS at room temperature for 5 min then twice with 0.1 × SSC plus 0.1% SDS at 68°C for 15 min. The hybridized probes were detected on X-ray film (Fuji Film) by the chemiluminescent reaction with Lumi-phos™530.

Genetic distance estimation and phylogenetic tree construction

The dissimilarities between chondriomes, calculated as 1-psb (the proportion of shared bands) for all possible pairs, were taken as the genetic distances. Two phylogenetic trees were constructed by the unweighted pair-group method using arithmetic averages (UPGMA) (Sokal and Sneath 1963) and the neighbor-joining (NJ) method (Saitou and Nei 1987). The software programs used were ulfah3 (N. Miyashita, unpublished) for genetic distance calculation and PHYLIP version 3.572 (Felsenstein 1993) for phylogenetic tree construction.

Results

RFLP analysis

Two examples of Southern hybridization patterns are shown in Fig. 2. Most of the patterns consist of a few major and some minor bands. Patterns of 2–18 bands were obtained using different probe-enzyme combinations (Table 3). Constitutions of all the band patterns are given in Appendix 1. Here, only the major bands were taken into account, because the minor bands may have resulted from hybridization of the probes with partially homologous sequences in the genome, plastome, or both (Nugent and Palmer 1991; Watanabe et al. 1994), or with subliminary fragments produced by intramolecular recombinations of mtDNA (Londsdale et al. 1984).

All of the probes and enzymes used detected strikingly large amounts of variation (Table 4); variable band frequencies were more than 90% in all cases. In total, there were 168 bands, of which 165 were variable (98.2%). The smallest number of different band patterns, 2, was obtained with *cox2-Pst*I; whereas, the largest number, 18, was obtained with the *cox1-Pst*I combination.

Classification of plasmon types using the Southern hybridization patterns of mtDNA

The band patterns obtained for each accession using the individual probe-enzyme combinations indicate that lines carrying the same type of plasmon had identical

Table 3 RFLP patterns of mtDNAs of lines (indcated by code numbers) bearing different plasmons, as obtained by each of 18 probe-enzyme combinations[a]

Plasmon type (code no.)	RFLP pattern[b]																	
	atpA-atp9		*atp6-rps13*		*cob*		*cox1*			*cox2*			*cox3*			*orf25*		
	Bm	Ps	Bm	Hn	Bm	Hn	Bm	Hn	Ps	Bm	Hn	Ps	Bm	Hn	Ps	Bm	Hn	Ps
A (01)	6	8	–	–	7	5	8	5	1	6	8	–	11	2	3	4	2	–
A^2 (16)	6	8	6	3	7	5	8	5	2	5	7	1	11	2	3	5	3	–
B (11,21,22,52,58)	8	6	5	–	3	4	6	10	11	4	9	2	5	5	6	3	8	3
C (02,27)	5	5	2	–	8	4	10	11	7	4	9	2	8	9	6	7	9	1
C^2 (38)	5	5	2	–	8	4	10	11	7	4	9	2	8	9	6	7	9	1
D-1 (04,19,28)	7	6	6	1	8	1	9	7	10	4	4	2	4	9	5	3	10	–
D-2 (36)	7	6	6	1	8	1	9	7	10	4	5	2	4	9	5	3	10	–
D^2-1 (35,55,56)	2	1	6	1	8	4	9	7	9	3	9	2	10	1	7	7	1	3
D^2-2 (53)	2	1	6	1	8	4	9	7	9	3	9	2	10	4	7	7	6	3
G (15,23,24,25,51)	3	8	2	4	8	3	10	11	4	4	9	2	5	5	6	1	7	6
G^2 (09)	3	8	2	4	8	3	10	11	4	4	9	2	5	5	6	1	7	6
M (05)	7	6	6	–	10	5	9	9	16	4	4	2	9	7	4	8	11	7
M^h (06)	4	7	4	1	10	5	2	6	5	2	5	2	7	9	6	7	9	5
M^o (31)	4	3	1	–	6	5	4	2	6	4	9	2	6	8	8	7	9	6
N (07)	1	2	6	4	3	1	1	11	18	4	4	2	7	9	6	2	5	4
S (08,17)	6	8	7	2	2	2	10	8	13	1	5	2	5	5	6	1	7	3
S^b (12)	7	2	6	4	8	5	5	11	15	4	2	2	3	9	1	6	4	2
S^l (10)	7	2	6	–	4	4	5	11	17	4	3	2	3	9	1	6	4	1
S^{l2} (20)	7	2	6	–	5	5	5	11	14	4	1	2	5	9	2	6	4	1
S^v (18,33,34,39)	7	2	6	4	8	5	5	11	15	4	1	2	3	9	1	6	4	2
T (13)	7	4	7	–	5	5	3	1	3	4	–	2	3	9	1	7	9	6
T^2 (14)	4	4	7	–	5	5	5	11	8	4	7	2	3	9	1	7	9	6
U-1 (03)	7	2	3	–	1	5	7	3	12	4	9	2	2	5	1	7	9	6
U-2 (26,29,37)	7	2	3	–	1	5	7	3	12	4	9	2	2	3	1	7	9	6
U-3 (32,54,57)	7	2	3	–	1	5	7	3	12	4	6	2	2	5	1	7	9	6
U^2 (30)	7	2	3	–	9	5	8	4	12	4	9	2	1	6	1	7	9	6

[a] The 6-base cutters, *Bam*HI, *Hin*dIII and *Pst*I, are abbreviated Bm, Hn and Ps

[b] Band constitutions of the RFLP patterns obtained with each probe-enzyme combination are given in Appendix 1

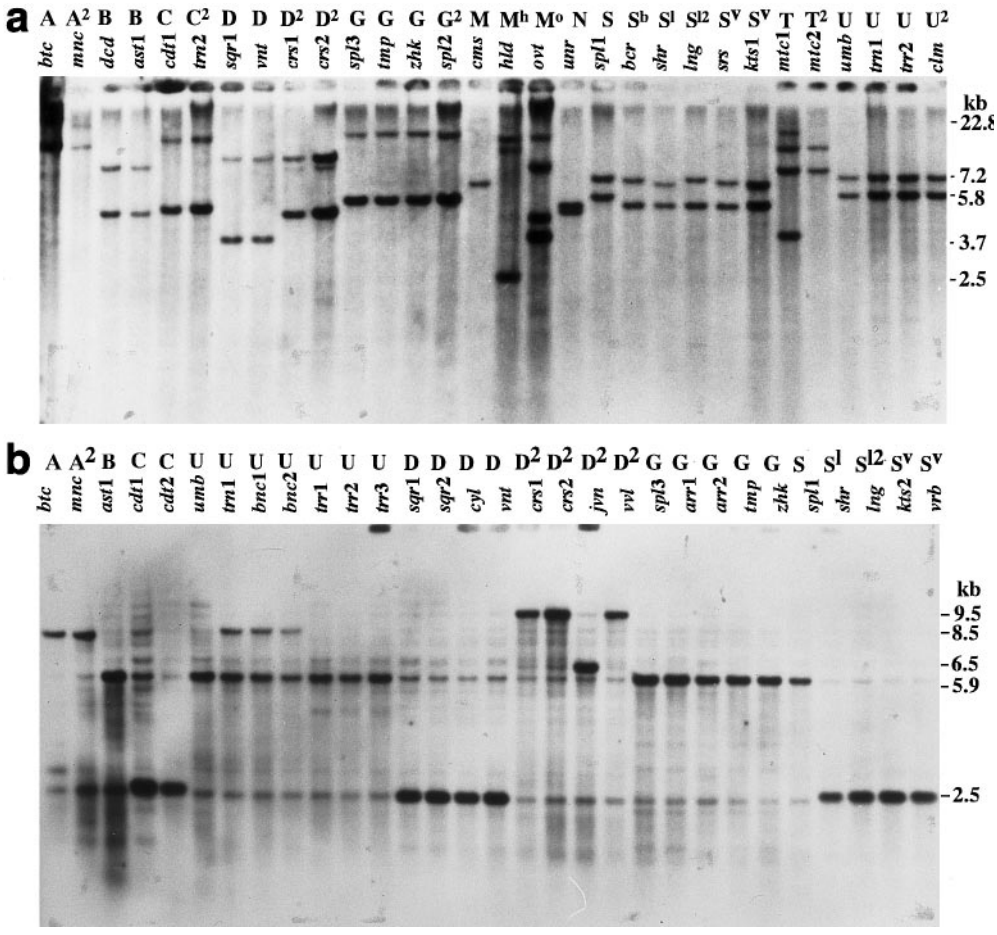

Fig. 2 a, b Southern hybridization band patterns of the total DNAs of 30 allo- and euplasmic lines of common wheat obtained by 2 mtDNA probe-restriction endonuclease combinations, *cox1-Pst*I (**a**) and *cox3-Hin*dIII (**b**). *Top* Code number and plasmon type of each line (ref. Table 1), *right* size (kb) of each molecular marker

Table 4 Numbers of total and variable bands (denominator and numerator, respectively, in column A) and the number of RFLP patterns (column B) detected by each probe-enzyme combination

Probe	Enzyme								Percentage RFLPs
	*Bam*HI		*Hin*dIII		*Pst*I		Total		
	A	B	A	B	A	B	A	B	
atpA-atp9	8/8	8	–	–	9/9	8	17/17	11	100
atp6-rps13	7/7	7	4/5	4	–	–	11/12	9	92
cob	11/11	10	5/5	5	–	–	16/16	14	100
cox1	11/11	10	15/15	11	24/24	18	50/50	19	100
cox2	8/9	6	8/9	9	2/2	2	18/20	13	90
cox3	10/10	11	7/7	9	8/8	8	25/25	14	100
orf25	8/8	8	14/14	11	6/6	7	28/28	15	100
Total	63/64	–[a]	53/55	–	49/49	–	165/168	23	–
Percentage RFLPs	98	–	96	–	100		–	–	98.2

[a] –: Not tested

band patterns for all the probe-enzyme combinations, whereas those carrying different types had different patterns for 1 or more probe-enzyme combinations (Table 3). There were two exceptional categories: (1) some lines assigned the same plasmon type showed different patterns for 1 or 2 probe-enzyme combinations, and (2) some lines of different plasmon types had the same patterns for all the probe-enzyme combinations.

Exceptions falling in the first category were found in the D, D^{2}and U plasmon types (Table 3, Appendix 1). Of the 4 lines bearing the D plasmon, line *vnt* (code no. 36) differed from lines *sqr*1, *sqr*2 and *cyl* (04, 19 and 28) in its *cox2-Hin*dIII pattern. Of the 4 lines bearing the D^2 plasmon, line *jvn* (code no. 53) differed in its *cox3-Hin*dIII and *orf25-Hin*dIII patterns from lines *crs*1, *crs*2 and *vvl* (code nos. 35, 55 and 56). In the third exception, of the 7 lines bearing the U plasmon, 3 lines *trr*1, *trr*2 and *trr*3 (code nos. 32, 54 and 57) differed from the rest in their *cox2-Hin*dIII pattern, and the 3 lines *trn*1, *bnc*1 and *bnc*2 (code nos. 26, 29 and 37) had a different *cox3-Hin*dIII pat-

Table 5 Average genetic distance between plasmon types (× 100)

Plasmon type	A	A^2	B	C /C^2	D -1	D -2	D^2 -1	D^2 -2	G /G^2	M	M^h	M^o	N	S	S^b	S^l	S^{l2}	S^v	T	T^2	U -1	U -2	U -3	U^2
A^2	25																							
B	78	86																						
C/C^2	73	77	67																					
D-1	79	77	64	62																				
D-2	79	77	64	62	4																			
D^2-1	85	83	71	65	50	50																		
D^2-2	85	82	69	63	47	47	8																	
G/G^2	74	73	57	59	70	70	72	71																
M	78	77	71	67	52	57	69	67	77															
M^h	83	85	75	56	61	58	61	62	73	52														
M^o	77	80	79	48	67	67	69	67	68	71	61													
N	84	80	58	55	54	58	70	70	60	65	67	74												
S	79	78	57	69	75	70	77	76	39	77	70	80	70											
S^b	73	71	67	45	54	54	65	65	60	55	67	57	50	74										
S^l	79	77	67	50	57	57	61	63	64	63	75	64	56	78	17									
S^{l2}	74	73	67	45	57	57	65	67	64	63	67	60	56	69	27	27								
S^v	73	71	67	45	54	54	65	65	60	55	67	57	50	74	5	17	22							
T	80	82	81	50	67	67	70	70	69	72	59	52	72	78	44	50	50	44						
T^2	74	74	77	43	64	64	67	64	65	69	58	38	67	74	38	48	48	38	24					
U-1	70	74	73	61	73	73	67	69	65	69	65	62	77	75	63	65	65	63	57	54				
U-2	66	70	73	62	73	73	67	70	66	70	66	62	77	75	64	66	66	64	58	55	2			
U-3	71	75	73	62	73	73	67	70	66	70	66	62	77	75	64	66	66	64	57	55	2	4		
U^2	69	73	72	56	67	67	66	68	64	68	61	61	71	74	52	56	60	52	48	45	15	17	17	
Average	74	75	70	58	61	61	65	65	66	67	66	65	66	72	54	58	57	53	61	57	59	59	60	57

tern than the other 4 lines. These exceptions may indicate that the mtDNA diffferences detected in the 3 plasmon types cause no remarkable phenotypic differences.

Two exceptions falling within the second category were found (Table 3 and Appendix 1). One was found in 2 lines, *cdt*1 and *cdt*2 (code nos. 02 and 27), carrying the C plasmon and 1 line (*trn*2; 38) carrying the C^2 plasmon. These lines produced identical band patterns with all of the probe-enzyme combinations, although their plasmon types differed. Similarly, 5 lines, *spl*3, *arr*1, *arr*2, *tmp* and *zhk* (code nos. 15, 23 24, 25 and 51) bearing the G plasmon and a single line (*spl*2; 09) bearing the G^2 plasmon gave identical band patterns with all the probe-enzyme combinations. These exceptions indicate that phenotypic differences observed between the C and C^2 plasmons and between the G and G^2 plasmons may be caused by molecular differences between their plastomes and/or other parts of their chondriomes not subjected to the present investigation (Wang et al. 1997).

In total, the chondriomes of 47 *Triticum* and *Aegilops* accessions, representing 22 plasmon types, could be classified into 24 types on the basis of the band patterns obtained by Southern hybridization.

Phylogenetic relationships between different chondriomes

The dissimilarities between the 23 chondriomes were calculated as 1-psb (the proportion of shared bands) for all of the possible pairs of chondriomes using the data in Table 3 and Appendix 1. The values obtained were regarded as the genetic distances between the chondriomes (Table 5). All lines bearing the same plasmon type had a zero distance, except for the D, D^2 and U plasmons (see above). Distances between the lines bearing 1 of these plasmons, however, were less than 0.10, indicative that their chondriomes are very similar. Close distances of less than 0.30 but more than 0.10 were found between lines bearing the A and A^2 plasmons, the S^b, S^l, S^{l2} and S^v plasmons, the T and T^2 plasmons and the U and U^2 plasmons, evidence that the chondriomes of all the subtype plasmons (A^2, S^{l2}, T^2 and U^2) are closely related to those of their respective main-type plasmons (A, S^l, T and U).

Phylogenetic trees of the chondriome constructed by the UPGMA and NJ methods are shown in Fig. 3. Three major groups, Einkorn, *Triticum* and *Aegilops*, were recognized at the distance of 0.65 or more (Fig. 3a). The Einkorn group has two diploid wheats, *T. boeoticum* and *T. monococcum*. The *Triticum* group has three subgroups, differing at a distance of more than 0.30: (1) two *Ae. speltoides* lines (code nos. 08 and 17), (2) Timopheevi wheats, including 2 *Ae. speltoides* lines (nos. 09 and 15) and (3) Emmer-Common wheats.

The *Aegilops* group has all the *Aegilops* species, except *Ae. speltoides*, and is divided into ten subgroups at a genetic distance of 0.30 or more (Fig. 3a): (1) 4 lines of *Ae. squarrosa*, *Ae. cylindrica* and *Ae. ventricosa* (all D plasmon), (2) 4 lines of *Ae. crassa*, *Ae. juvenalis* and *Ae. vavilovii* (all D^2 plasmon), (3) a single *Ae. uniaristata* line (N plasmon), (4) a single *Ae. comosa* line (M plasmon), (5) a single *Ae. heldreichii* line (M^h plasmon), (6) 7 lines of *Ae. searsii*, *Ae. kotschyi*, *Ae. variabilis* (all S^v

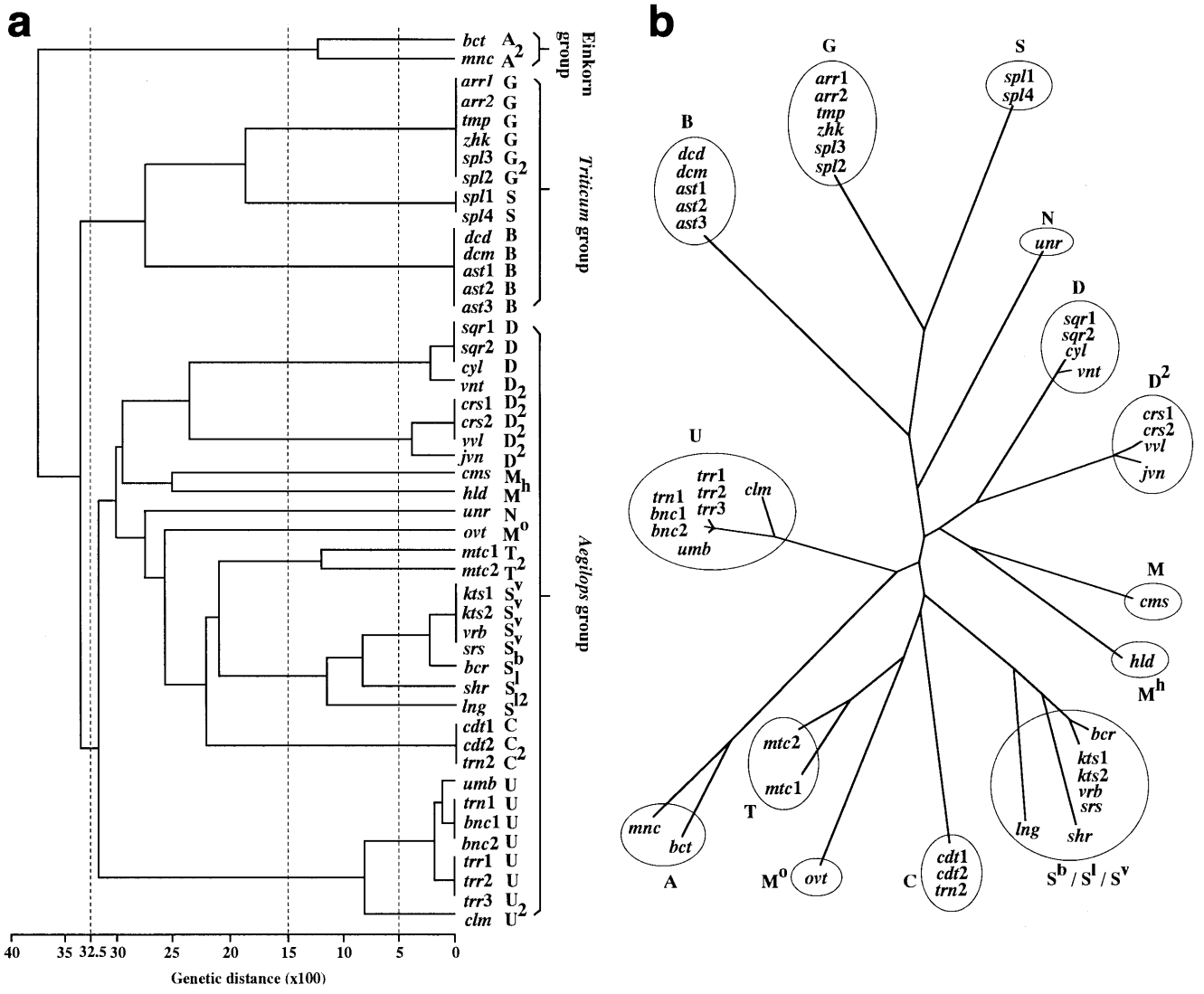

Fig. 3 a, b UPGMA (**a**) and NJ (**b**) trees for 47 chondriomes of *Triticum* and *Aegilops* based on the RFLP variation detected by the combination of seven mtDNA regions and three restriction endonucleases. Refer to Table 1 for abbreviations of the names of the lines and their plasmon types

plasmon), *Ae. bicornis* (S^b plasmon), *Ae. sharonensis* (S^l plasmon) and *Ae. longissima* (S^{l2} plasmon), (7) 3 lines of *Ae. caudata* (C plasmon) and *Ae. triuncialis* (C^2 plasmon), (8) 2 *Ae. mutica* lines (T and T^2 plasmon), (9) a single *Ae. ovata* line (M^o plasmon), and (10) 8 lines of *Ae. umbellulata, Ae. triaristata, Ae. trinucialis, Ae. biuncialis* (all U plasmon) and *Ae. columnaris* (U^2 plasmon). Within-subgroup variability was the largest in the sixth and eighth subgroups and second-largest in the tenth subgroup.

Discussion

Methodological features of the present investigation

Early studies of chondriome diversity in *Triticum* and *Aegilops* used the analysis of electrophoretic band patterns of endonuclease-treated mtDNAs isolated from intact mitochondria, here referred to as restriction pattern analysis (Vedel et al. 1978, 1981; Terachi and Tsunewaki 1986). This method provided information on the diversity of the entire chondriome. Because it requires a large amount of purified mtDNA, its application is limited to a small number of samples. As a result, the complete spectrum of chondriome diversity could not be obtained. Moreover, there were problems in homology determinations among the restriction fragments of different samples because the mtDNAs of *Triticum* and *Aegilops* produced very complex band patterns. Accurate identification of the homologous fragments therefore was possible only for mtDNA samples from different accessions of the same species or those of closely related species (Terachi and Tsunewaki 1986; Mori et al. 1995).

Table 6 Magnitudes of mtDNA variability among *Triticum* and *Aegilops* species detected by SSCP and RFLP analyses

MtDNA region	SSCP analysis[a]				RFLP analysis[b]			
	Probe size (kb)	Total no. bands	Numbers of variable bands	Percentage SSCP	Probe size (kb)	Total no. bands	Number of variable bands	Percentage RFLP
atpA-atp9	1.7	10	3	30	1.1	17	17	100
atp6-rps13	–	–	–	–	0.8	12	11	92
cob	0.6	2	0	0	0.6	16	16	100
cox1	1.5	9	5	56	1.5	50	50	100
cox2	1.9	13	6	46	1.9	20	18	90
cox3	0.7	4	0	0	0.7	25	25	100
orf25	0.4	3	3	100	0.4	28	28	100
Total	6.8	41	17	41.5	7.0	168	165	98.2

[a] A 4-base cutter, *Hin*fI, was used (after Wang et al. 1997)
[b] Three 6-base cutters, *Bam*HI, *Hin*dIII and *Pst*I, were used (present results)

The use of Southern hybridization analysis, in which small amounts of the total DNAs treated with the restriction enzyme were electrophoresed and hybridized with mtDNA probes after Southern transfer to a membrane, usually gives simple band patterns and allowed accurate identification of the homologous bands (Breiman 1987; Graur et al. 1989; Terachi and Tsunewaki 1992; Mori et al. 1995). One limitation of this method is its coverage of the entire chondriome. All the pioneering works used one to four mtDNA fragments as probes, with one to five restriction enzymes. These probes represented about one-sixtieth of the entire common wheat chondriome, which Quetier et al. (1985) had estimate to be approximately 430 kb. The total length of all the bands detected here in line *T. aestivum* (common wheat) using seven probes, however, was close to 80 kb (sum of a 9.6-kb *atpA-atp9-Pst*I fragment, a 3.7-kb *atp6-rps13-Bam*HI fragment, a 5.3-kb *cob-Bam*HI fragment, 8.8- and 4.9-kb *cox1-Pst*I fragments, 18.2-, 4.0- and 3.5-kb *cox2-Bam*HI fragments, 5.9- and 2.5-kb *cox3-Hin*dIII fragments and a 9.9-kb *orf25-Pst*I fragment; Appendix 1), about one-sixth of the entire wheat chondriome. We assume that similar coverage is achieved in other lines, although chondriomes may vary in size even within species (Fauron and Casper 1994).

As to the plant materials, most previous studies used only a few accessions of wheat and its close relatives (Vedel et al. 1978, 1981; Terachi and Tsunewaki 1986; Breiman 1987; Graur et al. 1989) or large numbers of accessions of a few species (Mori et al. 1995; Ohsako et al. 1996). Terachi and Tsunewaki (1992) made Southern hybridization analyses of the mtDNAs of 19 accessions belonging to 17 species using eight probe-enzyme combinations. This was up to the present the most comprehensive study carried out. The investigation reported here covers the widest range of the materials studied so far, with the 47 *Triticum* and *Aegilops* accessions used as mtDNA sources representing all the genome and plasmon types known in these genera. The number of probe-enzyme combinations used, 18, is also the largest of the reported studies. Our investigation should therefore provide the most comprehensive picture of the diversity and phylogenetic relationships of the chondriomes in *Triticum* and *Aegilops*.

A large part of mtDNA diversity is in the intergenic spacers

SSCP analysis detects both size and conformational differences among single-stranded mtDNAs within regions amplified by PCR. Conformational differences between single-stranded DNAs can not be detected by RFLP analysis. The use of a 4-base cutter in SSCP analysis facilitates the detection of very small mutations that may be overlooked in RFLP analysis. A higher sensitivity is therefore expected of SSCP than RFLP analysis in detecting molecular differences between mtDNAs.

Wang et al. (1997) conducted SSCP analyses of the mtDNAs of almost the same materials as those used in the present investigation. Their targets were nine mtDNA regions, six of which were used in the present RFLP analysis (Table 6). Although the total size of the mtDNA regions studied by Wang et al. (1997) and that of the probes used here correlated well, the relative frequencies of the variable bands (number of variable bands/total number of bands) were markedly higher in the RFLP (98.2%) than in the SSCP (41.5%) analysis, even though SSCP analysis should be more sensitive.

The SSCP analysis of Wang et al. (1997) detected only variation in PCR-amplified regions that consisted mainly of coding regions (Fig. 1). In contrast, RFLP analysis not only detected variation in these same regions but also variation in their flanking regions. The mtDNA variation detected by RFLP analysis therefore mostly exists in the intergenic spacers, and this method is concluded to be more efficient and useful than SSCP analysis for distinguishing the differences between chondriomes.

The high variability of the mtDNA detected by RFLP analysis may prove useful for plasmon identification. In fact, the chondriomes present in the 47 *Triticum* and *Aegilops* accessions that are representative of all the known plasmon types were classified into 24 groups us-

ing seven mtDNA probes with three restriction enzymes (Table 3). Plasmon types which could not be discriminated were limited to two pairs of plasmons; between C and C^2, and G and G^2. Except for these cases, all of the plasmon types could be distinguished using the minimum set of 3 probe-enzyme combinations *cox1-Pst*I, *cox2-Hin*dIII and *cox3-Bam*HI (Table 3). This minimum set can be used to identify most of the known plasmons and for preliminary screening of new plasmon types in *Triticum* and *Aegilops*.

Parallel evolutionary divergence of the chondriome and plastome

The most comprehensive data so far on plastome divergence in *Triticum* and *Aegilops* were reported by Ogihara and Tsunewaki (1988). They estimated the base substitution rates between all pairs of plastomes in 42 accessions, 39 of which were used in the investigation reported here. Their materials had 22 of the 24 chondriomes identified here. A correlation coefficient was calculated between the base substitution rates of the plastome and the genetic distances of the chondriome using the data of Ogihara and Tsunewaki (1988; ref. their Table 6) and of our study (Table 5). The coefficient, $r = +0.669$, with a degree of freedom $df = 229$, was significant at a probability of 0.001. This clearly indicates that diversification of the chondriome has paralleled that of the plastome during the evolution of *Triticum* and *Aegilops*.

Chondriome donors to individual groups of polyploids

As for the genus *Triticum*, chondriomes of the tetra- and hexaploid Timopheevi species were identical to those of 2 *Ae. speltoides* accessions ($d = 0.00$) but differed markedly from those of Einkorn wheat (d = 0.73–0.74) (Table 5, all hereafter), indicative that they received the chondriome from *Ae. speltoides*. All of the Emmer and Common wheats resembled *Ae. speltoides* much more (d = 0.57) than they did Einkorn wheat (d = 0.78–0.86) and other Sitopsis species (d = 0.67), indicative that their chondriomes also were derived from *Ae. speltoides* (ref. Fig. 3).

As for the Cylindropyrum section of *Aegilops*, a single polyploid species, *Ae. cylindrica*, was identical to *Ae. squarrosa*, the D-genome donor, but it differed markedly from *Ae. caudata*, the C-genome donor (d = 0.62); i.e., *Ae. squarrosa* was the chondriome donor to this tetraploid.

As for the polyploids of the Polyeides section, 1 accession of *Ae. triuncialis* and all of the accessions of *Ae. biuncialis, Ae. triaristata* (4 x and 6 x) and *Ae. columnaris* much more closely resembled *Ae. umbellulata*, the U-genome donor (d = 0.02–0.17), than did any of the diploids of the Comopyrum section, the modified M- or N-genome donor (d = 0.61–0.77), indicative that *Ae. umbellulata* was the chondriome donor to all of them. The other accession of *Ae. triuncialis*, however, was identical to *Ae. caudata*, the C-genome donor, but only distantly related to *Ae. umbellulata*, the U-genome donor (d = 0.61), evidence that its chondriome originated from *Ae. caudata*. *Ae. triuncialis* is the only species found to have originated from reciprocal crosses between the same two parents. *Ae. kotschyi* and *Ae. variabilits*, another polyploid group in this section, were identical to *Ae. searsii*, the modified S-genome donor, but differed markedly from *Ae. umbellulata*, the U-genome donor (d = 0.52–0.64), indicative that their chondriome donor was *Ae. searsii*. The remaining polyploid of this section, *Ae. ovata*, was only distantly related to both *Ae. umbellulata*, the U-genome donor, and all the diploids of the Comopyrum section, the possible modified M-genome donor (d = 0.61–0.74). Its closest diploid relative was an accession of *Ae. mutica*, a carrier of the T genome (d = 0.38) (Table 5). Thus, there is some doubt as to its chondriome donor.

As for polyploids of the Vertebrata section, *Ae. ventricosa* had a closer similarity to *Ae. squarrosa*, the D-genome donor (d = 0.04), than to *Ae. uniaristata*, the N-genome donor (d = 0.58), evidence that its chondriome originated from *Ae. squarrosa*. The 4 other polyploids of this section were clearly closer to *Ae. squarrosa*, the D-genome donor (d = 0.47–0.50), than to any of the diploids of the Comopyrum and Sitopsis sections, possible donors of the modified M, N or S genome (d = 0.61–0.77). This suggests that their chondriomes originated from *Ae. squarrosa* (Fig. 3).

Almost the same origins were assumed for the plastomes of all the polyploid species (Ogihara and Tsunewaki 1988), but the origin of the D^2 plasmon could not be assumed with certainty from plastome analysis. Both their results and ours taken together clearly show the plasmon donors and therefore the maternal parents of all the polyploids of *Triticum* and *Aegilops*, except that of *Ae. ovata*. We therefore can state that all the main problems regarding the maternal parents of the polyploid species in these genera have been solved, except for the origin of *Ae. ovata* (whether its modified M genome is related to the T genome of *Ae. mutica*, or whether its plasmon has been modified extensively since its origin).

On the assumption that the degree of chondriome variability between the species is proportional to the time of their plasmon divergence, the relative times of the origin of the polyploids can be estimated. Based on the present data, the divergence between the B plasmon of the Emmer-Common wheats and G/S plasmons of *Ae. speltoides*, and between the D^2 plasmon of *Ae. crassa* and its hexaploid allies and the D plasmon of *Ae. squarrosa*, are assumed to have occurred much earlier than the divergences between the plasmons of all the other polyploids and their plasmon donors. This speculation is supported in part by published molecular data on genome divergence (Zhang and Dvorak 1992; Dubcovsky and Dvorak 1995).

Wild species of both the Emmer and Timopheevi groups (*T. dicoccoides* and *T. araraticum*) had chondriomes identical to those of their respective cultivated

forms, *T. dicoccum* and *T. timopheevi*, indicative of recent domestication. As for the Einkorn group, *T. monococcum* is believed to be the cultivated form of *T. boeoticum*, not of *T. urartu* (Dvorak et al. 1988; Takumi et al. 1993). In our study, *T. boeoticum* and *T. monococcum* showed distinct chondriome differentiation. These facts suggest a much earlier domestication of Einkorn wheat than of Emmer and Timopheevi wheats.

Acknowledgements We thank T. Terachi, Kyoto Sangyo University, for his kind gift of the mitochondrial gene primers. We also thank N.T. Miyashita, Kyoto University, for providing the software program (ulfah3). GZW was supported by a Postdoctoral Fellowship for Foreign Researchers (ID No. P97471) from the Japan Society for the Promotion of Science.

Appendix

Appendix 1 Band constitutions of different RFLP patterns detected by 18 probe-enzyme combinations. Band size is given in kilobases

RFLP pattern	*atpA-atp9*		*atp6-rps13*		*cob*		*cox2*		
	*Bam*HI	*Pst*I	*Bam*HI	*Hin*dIII	*Bam*HI	*Hin*dIII	*Bam*HI	*Hin*dIII	*Pst*I
1	4.2	14.2+9.4	9.6	4.2+1.3	10.9+2.3	4.0	18.2+10.1+4.0 +3.5	20.1+9.0	14.1
2	4.0+2.3	14.2	6.9	4.0+1.3	6.2	2.2	18.2+5.5+4.0 +3.5	18.2+9.0	12.5
3	3.7+2.5	12.5+9.7	3.9	3.5+1.3	5.3	2.1+2.0	18.2+4.3+3.5	16.9+9.0	
4	3.5+2.3	12.5+8.7	3.8	3.2+1.3	4.9+4.2	2.0	18.2+4.0+3.5	13.2+9.0	
5	2.8+2.3	12.5+6.7	3.7		4.9+3.3	0.9	9.4+4.0+3.5 +2.0	11.8+9.0	
6	2.5	9.6	2.5		4.9+2.8		9.4+4.0+3.5 +1.6	9.6+9.0	
7	2.3	9.4+7.6	2.4		4.9+2.3			9.0+7.8	
8	2.0	3.1			4.9			9.0+3.1	
9					4.1+2.3			9.0	
10					4.0				

RFLP pattern	*cox1*			*cox3*			*orf25*		
	*Bam*HI	*Hin*dIII	*Pst*I	*Bam*HI	*Hin*dIII	*Pst*I	*Bam*HI	*Hin*dIII	*Pst*I
1	8.7	12.5+9.0+6.9 +3.0	22.8+11.7	17.4+9.0	9.5	9.7	4.9	9.6+3.0 +2.4	17.4
2	6.9+4.0+3.0	11.8+3.1+3.0	20.2+11.7	17.4	8.5+5.9	8.7	4.8	8.9+5.1	12.5
3	5.0+4.5	8.4+2.1+1.4	17.4+9.9 +7.9+3.7	9.0	8.5	7.9	4.6	8.9+1.6	9.9
4	5.0+3.2	8.4	15.8+5.8	8.8	6.5	7.4	2.5	7.5	9.6
5	5.0	7.5	14.1+9.9 +2.5	6.7	5.9+2.5	4.5	2.2	6.5	9.4+8.3
6	4.3	4.2+3.7+2.8	14.1+8.1 +4.5+3.8	5.2	5.9	3.8	2.1	5.8+3.0 +2.4	9.4
7	3.8+1.6	4.2	14.1+4.9	4.8	4.2	3.6	1.7	5.3+2.1	8.3
8	3.8	4.0	10.1+8.1	4.7	3.3	2.1	1.0	5.1	
9	3.0	3.7	9.4+4.7	4.5	2.5			2.4	
10	2.5	3.2	9.4+3.8	4.3				2.2+1.3	
11		3.0	8.8+4.9	3.8				2.0	
12			7.2+5.8						
13			7.0+5.3						
14			7.0+4.9						
15			6.9+4.9						
16			6.9						
17			6.8+4.9						
18			4.9+4.7						

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