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Chloroplast and nuclear microsatellite analysis of *Aegilops cylindrica*

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Abstract *Aegilops cylindrica* Host ($2n=4x=28$, genome CCDD) is an allotetraploid formed by hybridization between the diploid species *Ae. tauschii* Coss. ($2n=2x=14$, genome DD) and *Ae. markgrafii* (Greuter) Hammer ($2n=2x=14$, genome CC). Previous research has shown that *Ae. tauschii* contributed its cytoplasm to *Ae. cylindrica*. However, our analysis with chloroplast microsatellite markers showed that 1 of the 36 *Ae. cylindrica* accessions studied, TK 116 (PI 486249), had a plastome derived from *Ae. markgrafii* rather than *Ae. tauschii*. Thus, *Ae. markgrafii* has also contributed its cytoplasm to *Ae. cylindrica*. Our analysis of chloroplast and nuclear microsatellite markers also suggests that D-type plastome and the D genome in *Ae. cylindrica* were closely related to, and were probably derived from, the *tauschii* gene pool of *Ae. tauschii*. A determination of the likely source of the C genome and the C-type plastome in *Ae. cylindrica* was not possible.

Introduction

Jointed goatgrass (*Aegilops cylindrica* Host, $2n=4x=28$, genome CCDD) is an allotetraploid of the Triticeae tribe (Poaceae family). *Ae. cylindrica* is a close relative of wheat (*Triticum aestivum* L., $2n=6x=42$, genome AABBDD) and the two share the D genome (Riley and Law 1965; Kimber and Zhao 1983). This species is of worldwide economic importance for various reasons. First, jointed goatgrass is a widespread weed of bread wheat, chronically infesting fields in the Midwestern and western United States as well as fields in the Middle East and parts of Europe (Dewey 1996; Ogg and Seefeldt 1999; Guadagnuolo et al. 2001). Hybridization between jointed goatgrass and wheat and partial female fertility of the resulting naturally produced hybrids suggest the possibility of crop-to-weed gene movement (Zemetra et al. 1998; Morrison et al. 2002). Jointed goatgrass also has been identified as a source of useful genetic variation for wheat improvement (Farooq et al. 1992; El Bouhssini et al. 1998; Iriki et al. 2001). Therefore, there is considerable interest in understanding various aspects of the evolution of *Ae. cylindrica* for its better management and use.

Jointed goatgrass formed through amphidiploidization of a hybrid between *Ae. tauschii* Coss. ($2n=2x=14$, genome DD) and *Ae. markgrafii* (Greuter) Hammer (syn. *Ae. caudata* L., $2n=2 \ =14$; genome CC). This determination is based on data from a variety of sources including chromosome-pairing studies in interspecific hybrids (Kihara and Matsumura 1941; Kimber and Zhao 1983), karyotype analysis (Chennaveeraiah 1960), and analyses of protein and nuclear DNA variation (Jaaska 1981; Nakai 1981; Masci et al. 1992; Dubcovsky and Dvorak 1994). Furthermore, studies on phenotypic (Maan 1976; Tsunewaki 1996) and organellar DNA variation (Ogihara and Tsunewaki 1988; Wang et al. 1997, 2000a) established cytoplasmic homology between *Ae. cylindrica* and *Ae. tauschii* (D-type cytoplasm). These analyses suggested that *Ae. tauschii* was the maternal parent in the formation of *Ae. cylindrica*. However, studies on cytoplasmic variation in *Ae. cylindrica* have not been undertaken.

The nuclear genetic diversity of jointed goatgrass hasbeen studied using allozyme (Watanabe et al. 1994),

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C-banding (Badaeva et al. 2002), RAPD (Okuno et al. 1998; Goryunova et al. 2004), a combination RAPD and AFLP (Pester et al. 2003), and DNA sequence polymorphisms (Caldwell et al. 2004). These studies suggested that *Ae. cylindrica* had very low levels of genetic diversity, and that this allotetraploid originated recurrently. Although some studies indicated that the D genomes of wheat and *Ae. cylindrica* were apparently contributed by genetically distinct biotypes of *Ae. tauschii* (Badaeva et al. 2002; Caldwell et al. 2004), the relationship between *Ae. cylindrica* with subspecies of *Ae. tauschii* is not well defined. Similarly, the relationship between *Ae. cylindrica* and genetically differentiated populations of *Ae. markgrafii* (Ohta 2000, 2001) is unknown.

In this study, nuclear and chloroplast microsatellite markers were employed to investigate the relationships between *Ae. cylindrica* and its progenitors, *Ae. tauschii*, and *Ae. markgrafii*. This analysis and the new insights that it provides with respect to the evolution of *Ae. cylindrica* are discussed.

Materials and methods

Plant material

Chloroplast and nuclear microsatellite analyses were performed on 36 accessions of *Ae. cylindrica*, 17 accessions of *Ae. tauschii*, seven accessions of *Ae. markgrafii*, two accessions of *T. aestivum*, and two accessions of *T. turgidum*. The list of accessions along with their region of origin, the geographical coordinates of their collection site, and seed sources are presented in Table 1.

DNA isolation and molecular marker assays

The DNA was extracted from 35 mg of leaf tissue following the protocol described by Riera-Lizarazu et al. (2000). Twenty wheat chloroplast (WCt) microsatellite markers (Ishii et al. 2001; Table 2) were used to characterize the chloroplast genome and 19 Gatersleben wheat microsatellite (gwm) markers (Röder et al. 1998; Table 3) were used to evaluate the nuclear genome. For microsatellite marker assays, one primer was labeled with a fluorescent dye (6-carboxyfluorescein, or 4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein, or 4,7,2′,7′-tetrachloro-6-carboxyfluorescein). Polymerase chain reactions (PCRs) were carried out in 10-µl reactions comprising 0.03 U *Taq* polymerase with 1X PCR buffer containing 1.5 m *M* $MgCl_2$ (Qiagen, Valencia, Calif., USA), 2% sucrose in 0.04% cresol red, 0.2 m *M* of each dNTP, and 0.2 µ*M* of each primer. The PCR consisted of denaturation at 95° for 5 min, followed by 40 cycles of 95° for 1 min, 50–60° (depending on primers) for 1 min, and 72° for 1 min, with final extension at 72° for 10 min. Fragment analysis was carried out using an ABI Prism 377 DNA Sequencer and ABI Prism 3100 Genetic Analyzer. The ABI GeneScan, version 2.1, and Genotyper, version 2.0, software (Applied Biosystems, Foster City, Calif., USA) were used to size fragments based on an internal lane standard (*n*, *n*, *n*′, *n*′-tetramethyl-6-carboxyrhodamine or 6-carboxy-it *x*-rhodamine).

Spike morphology assessments

Spike and apical spikelet morphology can be used to distinguish *Ae. cylindrica* from its progenitors (Kimber and Feldman 1987; van Slageren 1994). Thus, spike morphology and the presence or absence of awns on apical glumes and lemmas were evaluated in some *Ae. cylindrica*, *Ae. tauschii*, and *Ae. markgrafii* accessions to verify their identities.

Cytological analyses

Root-tip collection, pre-treatment, and chromosome spread preparations for chromosome counting and karyotypic observations were carried out as described in Riera-Lizarazu et al. (1996). Slides were analyzed with a Zeiss Axiokop 2 (Carl Zeiss, Germany) microscope. Images were photographed with black-and-white Agfapan APX 100 film (Agfa-Gevaert, Mortsel, Belgium). Sample collection, treatments, and slide preparations for genomic in situ hybridization (GISH) performed on root-tip mitotic chromosome spreads of TK 116 were performed as described by Wang et al. (2002). *Ae. markgrafii* genomic DNA was used as the C-genome probe (biotinylated), and unlabeled *Ae. tauschii* genomic DNA was used as the D-genome hybridization competitor. Biotinylated DNA was detected with fluorescein conjugated Avidin, followed by signal amplification with biotinylated anti-avidin-D coupled with another layer of fluorescein-labeled Avidin. Unlabeled chromatin was counterstained with propidium iodide. Slides were analyzed with a microscope (Nikon Eclipse E1000) equipped with an epifluorescence attachment (with FITC, TRITC and dual-band FITC/PI filters; Chroma Technology, Brattleboro, VT). Images were taken with a built-in digital camera and were later processed using Adobe Photoshop, version 7.0 (Adobe Systems, San Jose, Calif., USA).

Statistical analyses

The number and frequency of alleles for each microsatellite marker were determined and used for the calculation of expected heterozygosity (Botstein et al. 1980). For both chloroplast and nuclear microsatellite markers, MICROSAT, version 2.0 (Minch et al. 1997), was used to generate a genetic distance (dissimilarity) matrix based on the proportion of shared alleles (Bowcock et al. 1994). The genetic distance matrix was then subjected to MEGA, version 2.0, for tree formation

Table 1 List of accessions along with their region of origin and the geographical coordinates of their collection sites

Species[a]	Accessions	Germplasm ID[b]	Region of origin	Geographical coordinates[c]	
				Latitude	Longitude
Aegilops markgrafii var. *markgrafii*	KU0006(A)	KU0006-2(A)	Syria	37.13	36.12
Ae. markgrafii var. *polyathera*	GR GB89	G591	Greece	NA	NA
Ae. markgrafii var. *markgrafii*	KU5472	KU5472	Iraq	35.54	44.84
Ae. markgrafii var. *polyathera*	KU5852(B)	KU5852(B)	Turkey	40.65	35.83
Ae. markgrafii var. *markgrafii*	KU5864 (C)	KU5864 (C)	Turkey	40.266	28.357
Ae. markgrafii var. *markgrafii*	KU5871(D)	KU5871(D)	Greece	NA	NA
Ae. markgrafii var. *markgrafii*	TK GB90	84TK159-036	Turkey	38.033	28.917
Ae. tauschii ssp. *tauschii*	AE1039/95	AE1039/95	Tadjikistan	NA	NA
Ae. tauschii ssp. *strangulata*	AE145/96	AE145/96	Azerbaijan	NA	NA
Ae. tauschii ssp. *strangulata*	AE184/78	AE184/78	Iran	NA	NA
Ae. tauschii ssp. *strangulata*	AE246/76	AE246/76	Uzbekistan	NA	NA
Ae. tauschii ssp. *tauschii*	AE257/76	AE257/76	Kyrgyzstan	NA	NA
Ae. tauschii ssp. *tauschii*	AE276/00	AE276/00	Afghanistan	NA	NA
Ae. tauschii ssp. *strangulata*	AE457/94	AE457/94	Georgia	41.69	44. 80
Ae. tauschii ssp. *strangulata*	AE498/79	AE498/79	Dagestan	NA	NA
Ae. tauschii ssp. *tauschii*	AE499/81	AE499/81	Turkmenistan	NA	NA
Ae. tauschii ssp. *tauschii*	G5792	G5792	China	NA	NA
Ae. tauschii	IRGB93	G1279	Iran	NA	NA
Ae. tauschii ssp. *tauschii*	TA10143	TA10143	Syria	35.31	38.45
Ae. tauschii ssp. *tauschii*	TA10144	TA10144	Syria	35.37	38.45
Ae. tauschii ssp. *tauschii*	TA10145	TA10145	Syria	35.37	38.45
Ae. tauschii ssp. *tauschii*	TA10146	TA10146	Syria	36.53	38.14
Ae. tauschii ssp. *tauschii*	TA1588	TA1588	Turkey	38.5	43.3
Ae. tauschii ssp. *tauschii*	TA2460	TA2460	Iran	NA	NA
Ae. cylindrica	AF 26	PI298891	Afghanistan	35.72	64.90
Ae. cylindrica	AR 147	IG48754	Armenia	39.83	44.83
Ae. cylindrica	AZ 133	IG48031	Azerbaijan	39.28	47.05
Ae. cylindrica	BG 137	IG48325	Bulgaria	42.02	23.65
Ae. cylindrica	DG 135	IG48260	Dagestan	41.93	48.37
Ae. cylindrica	GE 29	PI314406	Georgia	41.72	44.78
Ae. cylindrica	GR 159	PC	Greece	NA	NA
Ae. cylindrica	IQ 34	PI254864	Iraq	37.12	42.68
Ae. cylindrica	IR 149	IG48914	Iran	37.47	57.33
Ae. cylindrica	JO 146	IG48584	Jordan	31.78	36.80
Ae. cylindrica	LB 148	IG48789	Lebanon	34.47	36.33
Ae. cylindrica	SY 119	IG44621	Syria	33.92	36.70
Ae. cylindrica	TJ 142	IG48558	Tadjikistan	39.45	68.33
Ae. cylindrica	TK 1	PI172357	Turkey	40.27	40.25
Ae. cylindrica	TK 107	PI407639	Turkey	39.48	32.34
Ae. cylindrica	TK 115	PI554230	Turkey	37.13	44.52
Ae. cylindrica	TK 116	PI486249	Turkey	40.18	42.63
Ae. cylindrica	TK 120	IG47699	Turkey	40.23	28.20
Ae. cylindrica	TK 127	IG47906	Turkey	38.83	32.08
Ae. cylindrica	TK 129	IG47927	Turkey	38.97	35.60
Ae. cylindrica	TK 131	IG47959	Turkey	38.42	39.33
Ae. cylindrica	TK 14	PI542179	Turkey	39.35	26.75
Ae. cylindrica	TK 15	PI554201	Turkey	38.37	37.77
Ae. cylindrica	TK 16	PI486236	Turkey	37.30	44.57
Ae. cylindrica	TK 17	PI554206	Turkey	37.23	44.65
Ae. cylindrica	TK 19	PI554225	Turkey	38.40	42.60
Ae. cylindrica	TK 2	PI172358	Turkey	40.05	42.18
Ae. cylindrica	TK 39	G404	Turkey	36.85	40.05
Ae. cylindrica	TK 5	PI554203	Turkey	38.30	43.17
Ae. cylindrica	TM 139	IG48529	Turkmenistan	38.25	56.33
Ae. cylindrica	US/CO 61	PW27	USA	NA	NA
Ae. cylindrica	US/NE 45	PW6	USA	NA	NA
Ae. cylindrica	US/OR 13	FC13	USA	NA	NA
Ae. cylindrica	US/UT 21	FC21	USA	NA	NA
Ae. cylindrica	UZ 35	PI314185	Uzbekistan	41.37	69.55
Ae. cylindrica	YU 37	PI344778	Yugoslavia (Serbia)	44.02	20.92
T. turgidum ssp. *durum*	394	PI94705	Palestine	32.00	35.00

Table 1 (Contd.)

Species[a]	Accessions	Germplasm ID[b]	Region of origin	Geographical coordinates[c]	
				Latitude	Longitude
T. turgidum ssp. *durum*	Langdon	CItr 13165	USA	NA	NA
T. aestivum ssp. *aestivum*	Alcedo	TA 2933	Germany	NA	NA
T. aestivum ssp. *aestivum*	Chinese Spring	CItr 14108	China	NA	NA

[a]The variety (*Ae. markgrafii*) and subspecies (*Ae. tauschii*) designations are based on passport data, Pestova et al. (2000), Ohta (2000, 2001), and our own observations
[b]The first letter(s) of the germplasm ID makes reference to the sources of the germplasm. Accessions starting with *G* were obtained from Dr J. Giles Waines, University of California, Riverside, Calif., USA; *KU* accessions were obtained from Dr Shoji Ohta, Fukui Prefectural University, Japan; *AE* accessions were obtained from Institute of Plant Genetics and Crop Plant Research (IPK), Germany; *TA* accessions were obtained from Wheat Genetic Resource Center, Kansas State University, Manhattan, Kan., USA; *IG* accessions were obtained from the International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria; *CItr* and *PI* accessions were obtained from U.S. Department of Agriculture, National Small Grains Collection, Aberdeen, Idaho, USA; *FC*, *PW*, and *PC* (personal collections) accessions are maintained at Oregon State University, Corvallis, Idaho, USA
[c]Longitude and latitude coordinates are in the decimal system. *NA* indicates that the coordinates were not available

Table 2 Heterozygosity indices, number of alleles, and allele size range for *Ae. markgrafii*, *Ae. tauschii*, and *Ae. cylindrica*, using chloroplast microsatellite markers

Marker	*Ae. markgrafii*			*Ae. tauschii*			*Ae. cylindrica*[b]			TK 116
	No. of alleles	Allele size range (bp)	H^a	No. of alleles	Allele size range (bp)	H^a	No. of alleles	Allele size range (bp)	H^a	Allele size (bp)
WCt 1	2	113–114	0.24	2	111–112	0.21	2	110–111	0.11	112
WCt 2	2	124–125	0.49	5	128–131	0.65	3	128–130	0.36	124
WCt 3	4	151–159	0.69	4	147–154	0.53	2	146–147	0.45	156
WCt 4	3	193–198	0.61	2	193–197	0.57	1	196	0.00	197
WCt 5	2	81–82	0.49	4	81–84	0.63	3	82–84	0.56	83
WCt 6	1	187	0.00	4	184–188	0.66	2	186–187	0.16	187
WCt 8	1	148	0.00	2	148–149	0.11	3	147–149	0.21	147
WCt 9	1	120	0.00	1	120	0.00	1	120	0.00	120
WCt 10	2	194–195	0.49	3	192–194	0.46	2	192–193	0.16	195
WCt 11	3	167–169	0.61	5	166–170	0.78	2	166–167	0.24	166
WCt 12	2	146–147	0.49	4	148–151	0.67	2	149–150	0.06	146
WCt 13	1	104	0.00	3	105–107	0.55	2	104–106	0.16	104
WCt1 5	2	103–104	0.41	3	98–110	0.49	2	98–99	0.28	104
WCt 16	2	97–98	0.24	4	97–101	0.31	1	98	0.00	97
WCt 17	1	147	0.00	2	145–146	0.50	3	145–147	0.16	145
WCt 18	2	198–199	0.24	3	197–199	0.21	2	198–199	0.06	198
WCt 19	2	152–153	0.49	3	151–154	0.55	2	151–152	0.11	152
WCt 22	1	188	0.00	4	196–198	0.70	2	196–197	0.24	188
WCt 23	1	106	0.00	1	106	0.00	1	106	0.00	106
WCt 24	1	178	0.00	4	179–186	0.46	1	184	0.00	178
Average	1.8		0.28	3.15		0.45	1.95		0.17	

[a]The expected heterozygosity (*H*) was calculated as described by Botstein et al. (1980)
[b]Calculations did not include data from TK 116

(Kumar et al. 2001) using the neighbor-joining method (Saitou and Nei 1987). Tree View, version 1.6.6 (Page 2001), and MEGA were used to produce graphical outputs.

Results

For *Ae. cylindrica*, *Ae. tauschii*, and *Ae. markgrafii*, the average expected heterozygosity and number of alleles per marker were greater for nuclear than for chloroplast microsatellite markers (Tables 2, 3). Because there were only two genotypes each from *T. aestivum* and *T. turgidum*, their heterozygosity values were not calculated. The average expected heterozygosity for *Ae. cylindrica*, for both chloroplast and nuclear microsatellites, was lower than its progenitors, *Ae. markgrafii* and *Ae. tauschii*. For both chloroplast and nuclear microsatellite markers, *Ae. tauschii* showed the highest level of variation expressed as average expected heterozygosity and allele number per marker. Chloroplast markers with the highest average expected heterozygosity values were WCt 3 in *Ae. markgrafii* (0.69), WCt 11 in *Ae. tauschii* (0.78), and WCt 5 in *Ae. cylindrica* (0.56) (Table 2). The

Table 3 Heterozygosity indices, alleles, and allele size range for *Ae. markgrafii*, *Ae. tauschii*, and *Ae. cylindrica*, using nuclear microsatellite markers

Marker	*Ae. markgrafii*			*Ae. tauschii*			*Ae. cylindrica*		
	No. of alleles	Allele size range (bp)	H^a	No. of alleles	Allele size range (bp)	H^a	No. of alleles	Allele size range (bp)	H^a
gwm232	4	Null, 139–310	0.61	10	127–310	0.85	5	137–310	0.19
gwm337	2	Null, 166	0.41	12	152–213	0.89	6	164–193	0.41
gwm458	7	94–129	0.84	11	96–133	0.88	6	101–132	0.66
gwm642	8	169–191	0.83	18	108–200	0.88	5	170–187	0.70
gwm301	3	159–225	0.58	12	161–222	0.87	8	159–197	0.76
gwm455	4	120–133	0.61	9	128–188	0.76	3	127–187	0.10
gwm484	5	Null, 112–154	0.72	12	114–164	0.90	5	111–115	0.56
gwm608	7	110–134	0.84	3	101–110	0.54	1	110	0.00
gwm3	5	64–95	0.68	9	59–76	0.79	1	64	0.00
gwm314	2	Null, 99	0.24	17	Null, 99–268	0.94	10	171–183	0.83
gwm383	7	Null, 132–229	0.82	12	180–228	0.89	5	203–233	0.63
gwm186	5	Null, 95–147	0.78	4	Null, 96–169	0.56	3	Null, 98–99	0.35
gwm190	6	229–246	0.82	10	Null, 184–231	0.87	6	192–235	0.40
gwm205	3	133–136	0.57	5	127–310	0.70	4	129–147	0.28
gwm272	3	124–126	0.61	10	118–150	0.80	2	125–126	0.39
gwm325	3	Null, 114–127	0.65	7	114–142	0.81	2	113–114	0.05
gwm469	4	84–88	0.66	10	140–176	0.83	5	156–162	0.50
gwm437	3	Null, 159–165	0.53	11	83–129	0.86	5	Null, 87–99	0.59
gwm44	4	Null, 156–278	0.66	3	116–178	0.21	5	276–283	0.24
Average	4.47		0.66	9.74		0.78	4.58		0.40

[a] *H* was calculated as described by Botstein et al. (1980)

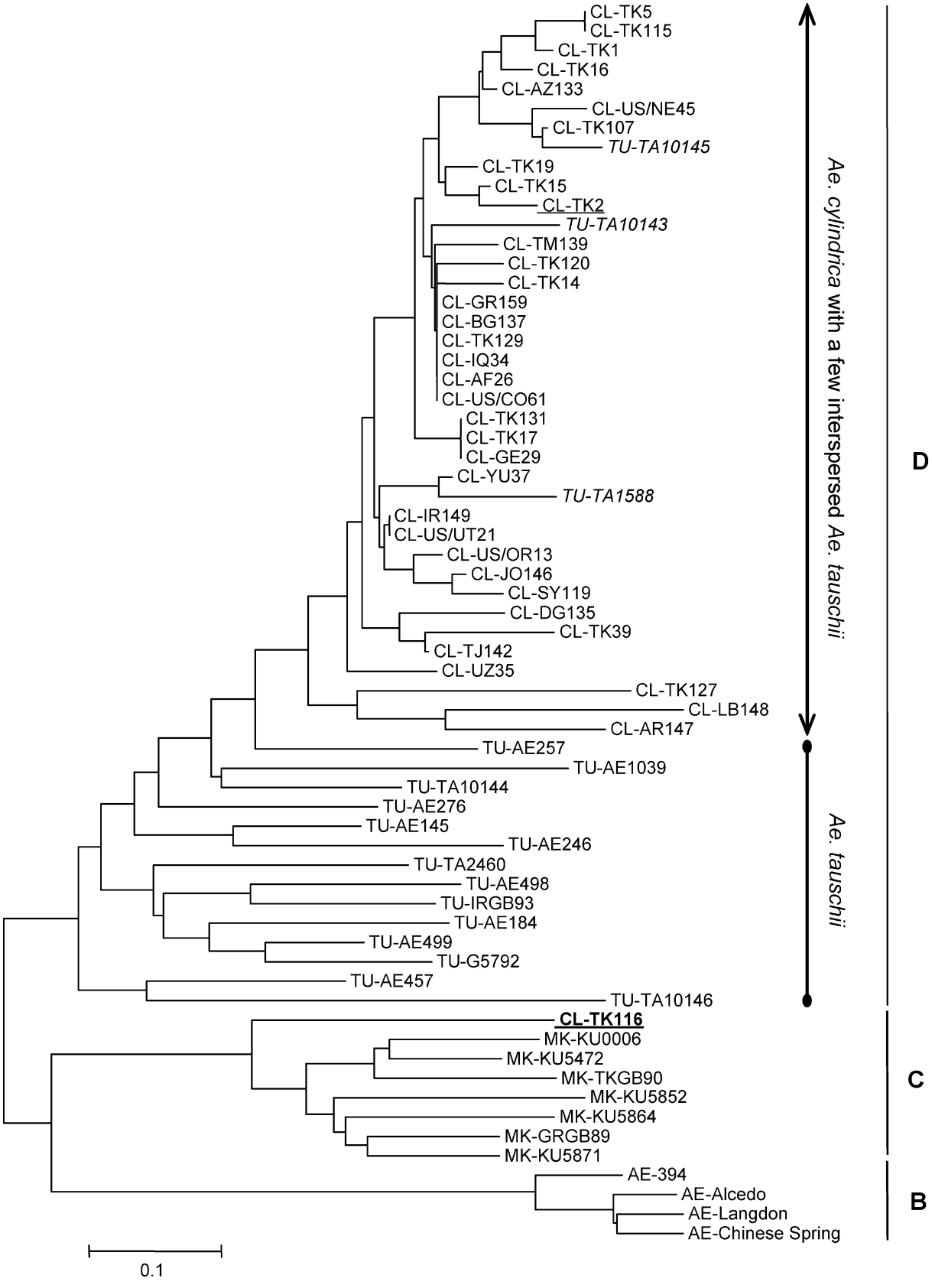
nuclear marker gwm458 showed the highest heterozygosity in *Ae. markgrafii* (0.84), while nuclear marker gwm314 showed the highest heterozygosity in *Ae. tauschii* and *Ae. cylindrica* (0.94 and 0.83, respectively) (Table 3).

Based on genetic similarity analysis with 20 chloroplast microsatellite markers, genetic distance between any two accessions ranged from 0 (most similar) to 0.9 (most dissimilar). This analysis also allowed the distinction of species with respect to various plastome types. Seven markers (WCt 1, WCt 2, WCt 12, WCt 13, WCt 17, WCt 22, and WCt 24) permitted the differentiation of 64 accessions from five species into plasmon types B, C, and D (Fig. 1). An unanticipated finding was that one accession of *Ae. cylindrica*, TK 116 (PI 486249), exhibited some microsatellite alleles that were present neither in *Ae. cylindrica* nor in *Ae. tauschii* accessions but matched the allelic constitution of some *Ae. markgrafii* accessions (Table 2).

Thirty-five *Ae. cylindrica* and 17 *Ae. tauschii* accessions formed a single major cluster (D-type plastome). Of the 17 *Ae. tauschii* accessions studied, 14 formed a dispersed group while three accessions (TA 1588, TA 10143, and TA 10145) intermingled with *Ae. cylindrica*. The *Ae. cylindrica* accession TK 2 (PI 172358), which had been previously used to determine that *Ae. cylindrica* had plasmon type D (Maan 1976) fell in this major cluster with other *Ae. cylindrica* accessions. The wheat lines Chinese Spring, Alcedo, Langdon, and 394, and seven *Ae. markgrafii* accessions were part of a cluster with two distinct groups (B-type and C-type plastomes). One *Ae. cylindrica* accession, TK 116, grouped with *Ae. markgrafii*. This was consistent with our observation that the allelic constitution of this accession was more similar to *Ae. markgrafii* than *Ae. tauschii*.

Nineteen nuclear microsatellites were also used to study the genetic relatedness of *Ae. tauschii*, *Ae. markgrafii*, and *Ae. cylindrica* accessions. The genetic distances ranged from 0.05 (most similar) to 0.98 (most dissimilar). The 65 accessions studied grouped into two major clusters (Fig. 2). *Ae. cylindrica*, *Ae. markgrafii*, and nine *Ae. tauschii* accessions grouped in cluster I, whereas tetraploid and hexaploid wheat and eight *Ae. tauschii* accessions grouped in cluster II (Fig. 2; Table 3). Cluster I was subdivided into a group with *Ae. cylindrica* and *Ae. markgrafii* accessions (group CM) and a group of nine *Ae. tauschii* accessions (group TU1). The CM group was composed of *Ae. cylindrica* (group CL), five *Ae. markgrafii* accessions (group MK), and two other *Ae. markgrafii* accessions that grouped between *Ae. markgrafii* and *Ae cylindrica* (Fig. 2). TK 116 was present in the CL group. The *Ae. markgrafii* accessions KU 5472 and TK GB90 were most closely related to *Ae. cylindrica* (Fig. 2). Cluster II was subdivided into a group represented by tetraploid wheat (group DU), a group with hexaploid wheat and six *Ae. tauschii* accessions (group TU2), and two other *Ae. tauschii* accessions (Fig. 2).

Spike morphology and cytological analyses were also conducted to investigate the identity of TK 116. The apical spikelets of *Ae. cylindrica* have four prominent awns, with one pair originating from glumes and one pair from lemmas of the apical spikelet (van Slageren 1994). On the other hand, apical spikelets of *Ae. mark-*



grafii have two prominent awns coming from the apical glumes, whereas apical spikelets of *Ae. tauschii* have two awns originating from two lemmas. In the present study, similar characteristics were noted for *Ae. markgrafii* and *Ae. tauschii* (Fig. 3). The spikes of TK 116 and another *Ae. cylindrica* accession (USA/OR 13) have a cylindrical

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Fig. 1 Neighbor-joining tree showing chloroplast genetic relatedness between *Aegilops cylindrica* and its relatives. TK 116 and TK 2 (an accession used in alloplasmic interaction studies) are *underlined*. *Ae. tauschii* accessions interspersed with *Ae. cylindrica* are *italicized*. The prefixes used before the name of each accession stand for the following: *AE T. aestivum*, *CL Ae. cylindrica*, *DU Triticum turgidum*, *MK Ae. markgrafii*, and *TU Ae. tauschii*. Clusters of accessions designated as *B*, *C*, and *D* correspond to individuals with plasmon types B, C, and D, respectively

structure and bear four prominent awns on glumes and lemmas from apical spikelets. The overall similarity of TK 116 with other *Ae. cylindrica* accessions with respect to spike morphology and the number of awns in apical spikelets supports its classification as an *Ae. cylindrica* accession. Based on chromosome counting and GISH analysis, TK 116 was found to be a 28-chromosome allotetraploid with both C-genome and D-genome chromosomes (Fig. 4a, b).

Discussion

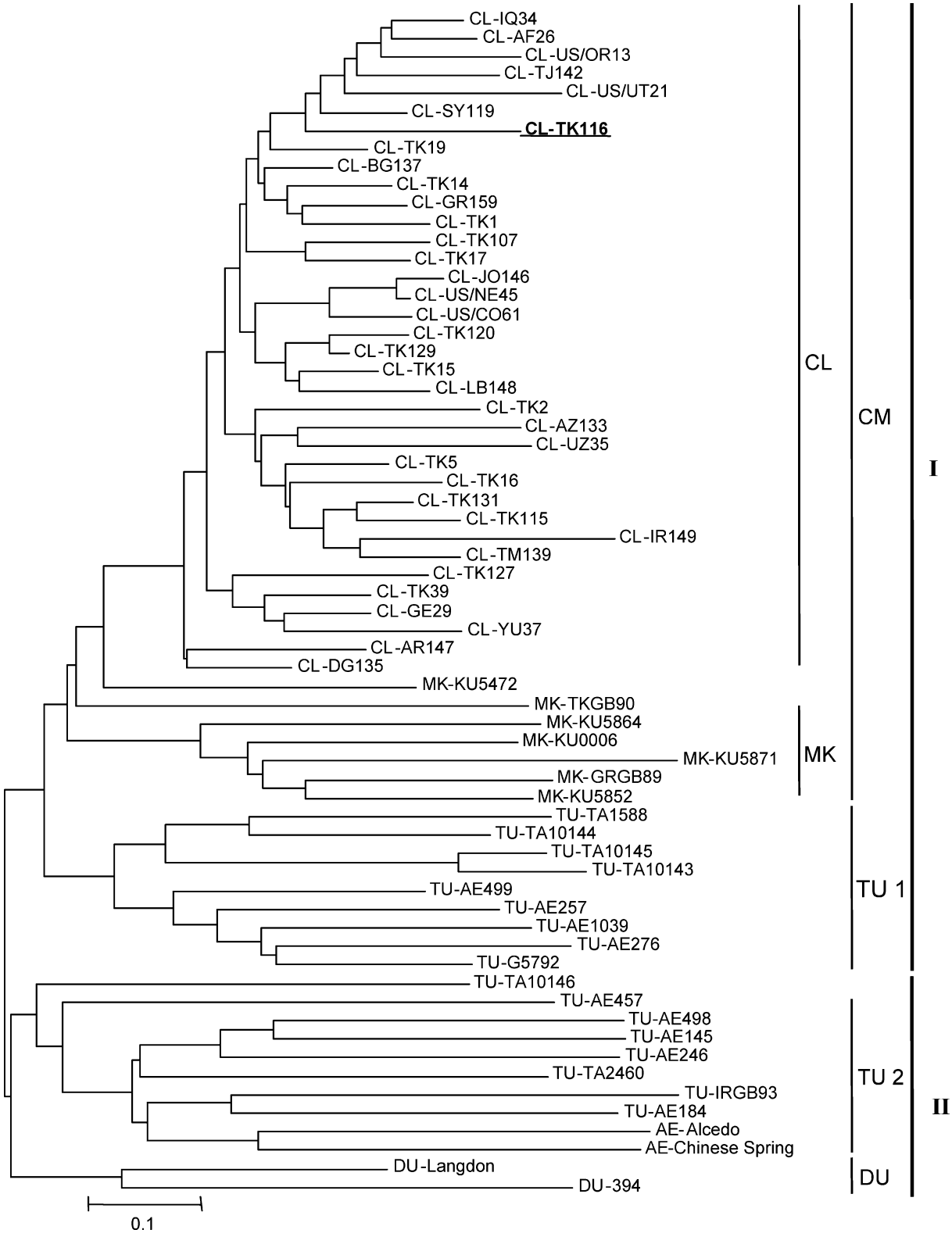
The evaluation of both chloroplast and nuclear microsatellite variation revealed various patterns (Tables 2, 3). First, the level of chloroplast variation compared to nuclear variation was lower for all species studied. The lower levels of variation in chloroplast compared to nuclear microsatellites reflect the uniparental inheritance of chloroplast genomes and their slower rate of evolution relative to nuclear genomes (Wolfe et al. 1987; Provan et al. 1999, 2004). Second, *Ae. cylindrica* was less diverse than either of its diploid progenitors (*Ae. markgrafii* and *Ae. tauschii*) whether chloroplast or nuclear markers were used. Because allopolyploids are formed from one or few relatively recent hybridization events, these contain only a subset of the genetic variation present in their progenitors. Thus, allopolyploids like *Ae. cylindrica* are commonly less diverse than their progenitors. Third, *Ae. tauschii* was more diverse than *Ae. markgrafii*. Goryunova et al. (2004) also made this observation and suggested that this reflected a more ancient origin for *Ae. tauschii* relative to *Ae. markgrafii*. Although our observations are consistent with those of Goryunova et al. (2004), a larger sampling of *Ae. markgrafii* accessions will be needed to fully address this difference in genetic diversity. Finally, *Ae. cylindrica* was more closely related to *Ae. markgrafii* than *Ae. tauschii* when nuclear microsatellites were analyzed. The close relationship between *Ae. cylindrica* and *Ae. markgrafii* was also observed using repetitive DNA markers (Dubcovsky and Dvorak 1994), RAPD markers (Goryunova et al. 2004), and analysis of the internal transcribed spacers of ribosomal RNA genes (Wang et al. 2000b). These observations demonstrate that the C genome in *Ae. cylindrica* is less divergent from the C genome of *Ae. markgrafii* than its D genome is from the D genome of *Ae. tauschii*.

Plasmon analysis using wheat alloplasmic lines indicated that *Ae. tauschii* (D-type cytoplasm) was the maternal parent in the formation of *Ae. cylindrica* (Tsunewaki 1996; Wang et al. 1997, 2000a). However, our current investigation showed that one accession of *Ae. cylindrica*, TK 116 (PI 486249), had chloroplast microsatellite alleles that were present neither in *Ae. cylindrica* nor in *Ae. tauschii* accessions but matched the allelic constitution of some *Ae. markgrafii* accessions (Table 2; Fig. 1). This finding suggested that the chloroplast genome of TK 116 was derived from *Ae. markgrafii* (C-type cytoplasm). Because our nuclear microsatellite markers analysis (Fig. 2), spike morphology assessments (Fig. 3), and karyotype evaluations (Fig. 4) showed that TK 116 was a bona fide *Ae. cylindrica* accession, we conclude that C- and D-types of cytoplasm derived from *Ae. markgrafii* and *Ae. tauschii*, respectively, are present in *Ae. cylindrica*.

We contemplated the possibility that our results with respect to TK 116 could be explained by chloroplast microsatellite allele size homoplasy (Doyle et al. 1998; Hale et al. 2004). However, we reasoned that this was unlikely, because we evaluated a sizeable number of accessions with 20 chloroplast microsatellite markers. Other researchers also have found that homoplasy was unlikely for chloroplast markers when evaluating closely related genera, including species of the Triticeae, due to their relatively slow rate of evolution compared to nuclear loci (Provan et al. 2004).

The occurrence of two types of cytoplasm in *Ae. cylindrica* may be simply explained by reciprocal hybridization between *Ae. markgrafii* and *Ae. tauschii* during the formation of *Ae. cylindrica*. Because reciprocal hybrids between *Ae. tauschii* and *Ae. markgrafii* have been produced experimentally (Sears 1941; Knobloch 1968), it is plausible that reciprocal hybridization in nature led to the formation *Ae. cylindrica* with both C-type and D-type cytoplasm. Interestingly, reciprocal hybridization between *Ae. markgrafii* and *Ae. umbellulata* Zhuk. ($2n = 2x = 14$, UU) has also been proposed in the origin of the allotetraploid species *Ae. triuncialis* L. ($2n = 4x = 28$, genome CCUU) (Murai and Tsunewaki 1986; Wang et al. 1997; Vanichonon et al. 2003). Because evidence for multiple hybridization events in the formation of *Ae. cylindrica* has been recently presented by Caldwell et al. (2004), reciprocal hybridization is an attractive mechanism to explain the presence of C-type and D-type plastomes in this species. However, cytoplasmic introgression or substitution should also be considered (Rieseberg and Soltis 1991; Brubaker et al. 1993; van Raamsdonck et al. 1997). In this scenario, hybridization between *Ae. markgrafii* (female parent) and *Ae. cylindrica* (male parent) followed by backcrossing with *Ae. cylindrica* (male parent) would also result in *Ae. cylindrica* with C-type cytoplasm (Kihara and Matsumura 1941).

Based on a comprehensive survey of *Ae. tauschii* germplasm with nuclear DNA markers, Dvorak et al. (1998) suggested that the distribution of present-day *Ae. tauschii* originated by expansion of two geographically isolated subspecies— *Ae. tauschii* ssp. *strangulata* in coastal areas of eastern Caspian Iran and ssp. *tauschii* in



an inland area of northwestern Iran. According to Dvorak et al. (1998), expansion of the distribution of ssp. *tauschii* preceded that of ssp. *strangulata* leading to the spread of ssp. *tauschii* westward to Turkey and eastward to Afghanistan, Turkmenistan, Pakistan, Tadjikistan, and China. Subsequently, expansion of the distribution of ssp. *strangulata* and gene flow between the subspecies in the Caspian region and north-central Iran was argued to have resulted in the observed discontinuity in the distribution of ssp. *tauschii* in Iran

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Fig. 2 Neighbor-joining tree showing the nuclear genetic relatedness between *Ae. cylindrica* and its relatives. TK 116 is *underlined* and in *boldface*. The two major clusters are labeled as *I* and *II*. Based on membership, major clusters were subdivided into groups labeled *CM* (*Ae. cylindrica* and *Ae. markgrafii*), TU1 (*Ae. tauschii*), TU2 (*Ae. tauschii* and *T. aestivum*) and DU (*T. turgidum*). The CM sub-cluster was further split into the CL (*Ae. cylindrica*) and MK (*Ae. markgrafii*) groups. *Ae. tauschii* accessions in the TU1 grouping belong to the *tauschii* gene pool while *Ae. tauschii* in the TU2 group belong to the *strangulata* gene pool. The prefixes used before the name of each accession stand for the following: *AE T. aestivum*, *CL Ae. cylindrica*, *DU T. turgidum*, *MK Ae. markgrafii*, and *TU Ae. tauschii*

(Lubbers et al. 1991; Dvorak et al. 1998). Furthermore, Dvorak et al. (1998) suggested that *Ae. tauschii* germplasm should be viewed as being composed of two gene pools, *strangulata* and *tauschii*, rather than two subspecies based on morphology. Nonetheless, this and other studies have shown that the D genome in hexaploid wheat is more closely related to the D genome of the *strangulata* gene pool of *Ae. tauschii* (Lubbers et al. 1991; Dvorak et al. 1998; Pestsova et al. 2000).

Based on our analysis of nuclear microsatellite markers, *Ae. tauschii* clustered in two distinct groups (TU1 and TU2) (Fig. 2). The TU2 group was composed of *Ae. tauschii* and hexaploid wheat (Alcedo and Chinese Spring). *Ae. tauschii* accessions in the TU2 group belong to the *strangulata* gene pool, whereas the *Ae. tauschii* accessions in the TU1 group that are more closely related to *Ae. cylindrica* belong to the *tauschii* gene pool (Table 1) (Dvorak et al. 1998; Pestova et al. 2000).

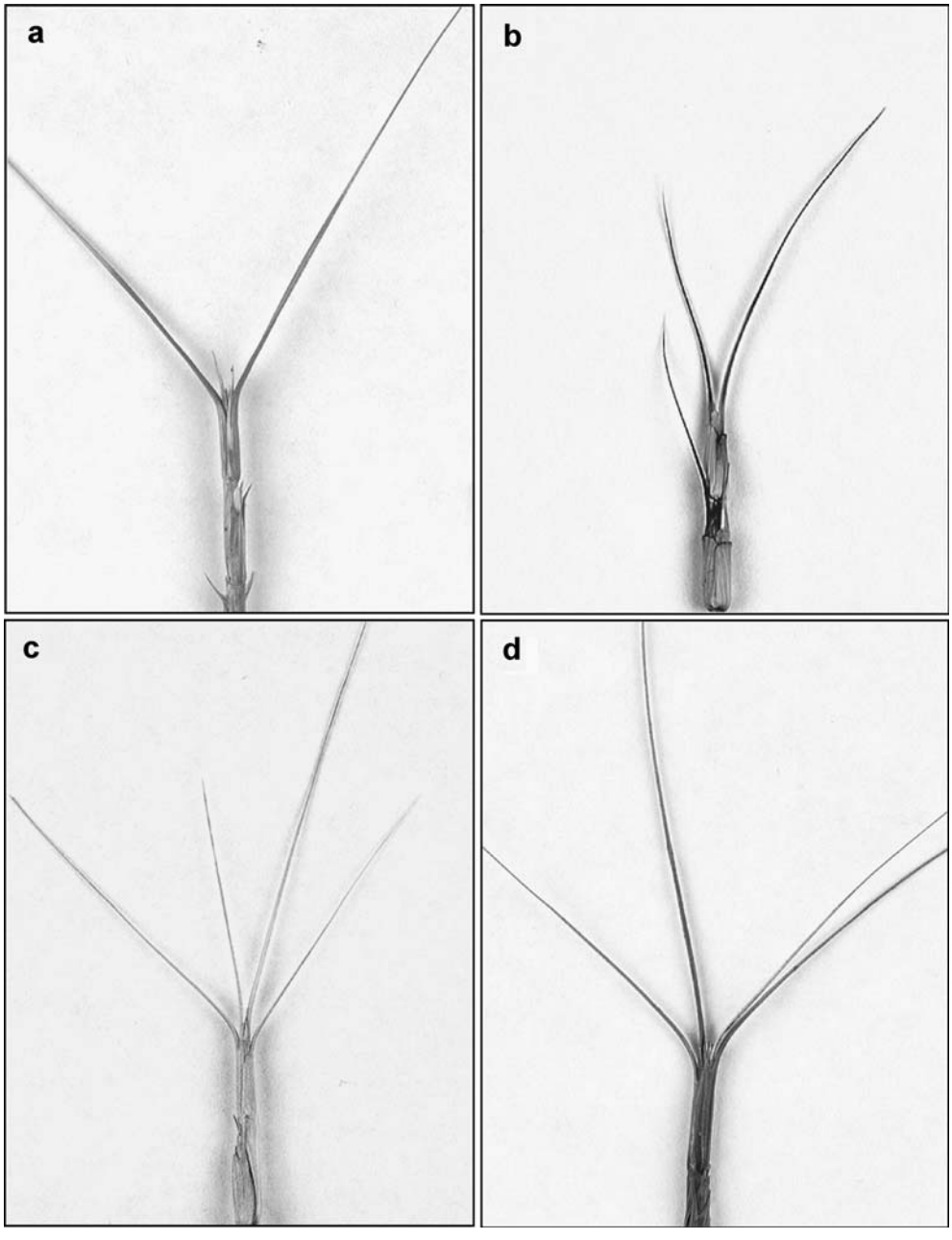

Fig. 3 Apical portions of spikes from *Ae. markgrafii*, *Ae. tauschii*, and *Ae. cylindrica*. **a** Apical spikelet of the *Ae. markgrafii* accession GR GB89 showing two awns originating from the apical glumes. **b** Apical spikelet of the *Ae. tauschii* accession AE 276 showing two awns originating from two apical lemmas. **c**, **d** Apical spikelets of the *Ae. cylindrica* accessions US/OR 13 and TK 116, respectively. Apical spikelets in (**c**) and (**d**) show four awns originating from both lemmas and glumes

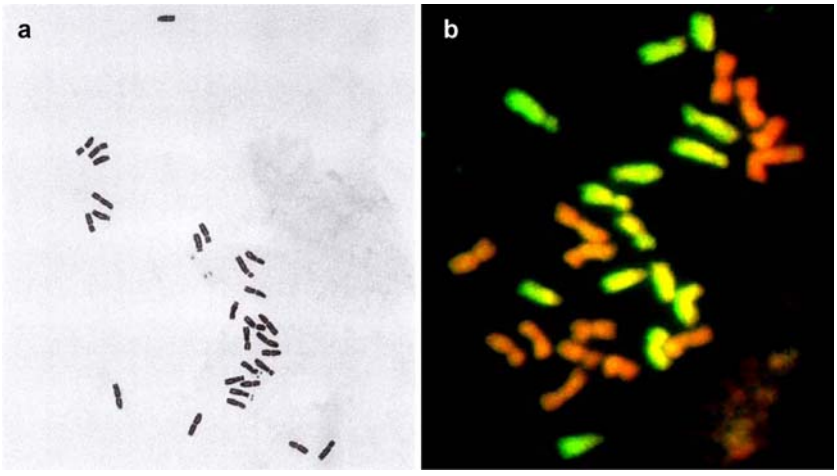

Fig. 4 Mitotic metaphase chromosome spreads and genomic in situ hybridization (GISH) of the *Ae. cylindrica* accession TK 116 (PI 486249). **a** Chromosome spread of TK116 showing 28 chromosomes with a combination of chromosomes with terminal, subterminal, sub-median, and median centromeres. **b** GISH of a mitotic chromosome spread of TK116. Fourteen fluorescein-labeled chromosomes (*yellow-green*) correspond to C-genome chromosomes while 14 *red-orange* (propidium iodide)-colored chromosomes correspond to D-genome chromosomes

Furthermore, three accessions of the TU1 group (TA 1588, TA 10143, and TA 10145) were interspersed with *Ae. cylindrica* in the dendrogram based on chloroplast microsatellite data (Fig. 1). Overall, this suggests that the D genome and D-type plastome in *Ae. cylindrica* are closely related to and were probably derived from the *tauschii* gene pool of *Ae. tauschii*. This conclusion is consistent with molecular cytogenetic analyses showing that D-genome chromosomes in *Ae. cylindrica* and common wheat were derived from different *Ae. tauschii* biotypes (Badaeva et al. 2002).

Based on spike morphology, two taxonomic varieties of *Ae. markgrafii* have been described (Eig 1929; Hammer 1980). Variety *typica* (syn. *Ae. markgrafii* var. *markgrafii*) is characterized by well-developed awns on apical glumes and awnless glumes of lateral spikelets while var. *polyathera* (syn. *Ae. markgrafii* var. *polyathera*) has awned apical and lateral spikelets. Irrespective of this varietal differentiation, studies on intraspecific hybrid sterility and the genetic variation for the development of awns on lateral spikelets suggested that *Ae. markgrafii* is divided into two genetically differentiated populations (Ohta 2000, 2001). One population is present in the western region encompassing Greece and West Anatolia, whereas the other population is present in the eastern region consisting of central, southern, and eastern Anatolia, Syria, and northern Iraq.

In our analysis with chloroplast and nuclear markers, the genetic differentiation of *Ae. markgrafii* accessions from the west and east was not evident. The *Ae. markgarfii* accessions KU 0006 (*typica* from northwestern Syria), KU 5852 (*polyathera* from north-central Turkey), KU 5864 (*typica* from northwestern Turkey), and KU 5871 (*typica* from mainland Greece) formed a single group (MK) in our dendrogram based on nuclear markers (Fig. 2). On the dendrogram-based on chloroplast markers, KU 5852, KU 5864, and KU5871 formed a sub-group, whereas KU 0006 associated with other *Ae. markgrafii* accessions (Fig. 1). Thus, KU 0006 and KU 5852 that correspond to Ohta's (2000) A and B testers of the eastern region and KU 5864 and KU 5871 that correspond to the C and D testers of the western region, respectively, were all closely related. This inability to differentiate *Ae. markgrafii* genotypes from the west from those of the east did not allow the identification of a probable source for the C genome or C-type plastome in *Ae. cylindrica*. The two *Ae. markgrafii* accessions most closely related to *Ae. cylindrica* based on nuclear markers were a *typica* form the east, KU 5472 (from northern Iraq), and *typica* from the west, TK GB90 (from western Turkey) (Fig. 2).

Maps with collection sites of *Ae. markgrafii* and *Ae. tauschii* suggest that the geographic distribution of these species overlap in southeastern Turkey, northeastern Syria, northern Iraq, and northwestern Iran (van Slageren 1994; Ohta 2000; Dvorak et al. 1998). Assuming that the distributions of these species were not significantly different in the past, then the central part of the Fertile Crescent is likely to be where *Ae. cylindrica* formed. Our observation that *Ae. tauschii* of their western region of distribution (*tauschii* gene pool) are most closely related to *Ae. cylindrica* is consistent with this hypothesis. However, this pattern was not evident with the sample of *Ae. markgrafii* that we used. An analysis of a more comprehensive sample of *Ae. markgrafii* accessions and an assessment of the population structure of this species may be necessary before a connection to *Ae. cylindrica* is possible. Similarly, a study with a larger sample of *Ae. cylindrica* and its progenitors may be necessary to obtain a more precise picture of these genetic and geographical connections.

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Conflict of interest: No information supplied

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