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ORIGINAL PAPER

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Genealogical use of chloroplast DNA variation for intraspecific studies of *Aegilops tauschii* Coss.

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Abstract Intraspecific patterns of chloroplast DNA variation was studied in *Aegilops tauschii* Coss., the D-genome progenitor of bread wheat. Nucleotide sequences of ten chloroplast microsatellite loci were analyzed for 63 accessions that cover the central part of the species distribution. As is often the case with nuclear microsatellites, those of chloroplasts of *Ae. tauschii* bear complex mutations. Several types of mutations other than change in the microsatellite repeat number were found, including base substitutions and length mutations in flanking regions. In total, eight mutations were present in the flanking regions of four loci. Most mutations in the flanking regions of microsatellite repeats are associated with biallelic polymorphisms. Phylogeographic analyses showed that such biallelic polymorphisms are useful to investigate intraspecific patterns of monophyletic lineage divergence. In contrast, most microsatellite repeat sites are multiallelic, variable within intraspecific lineages, and useful to compare degrees of genetic diversity between lineages. These findings show that the chloroplast genome harbors evolutionary variations informative for intraspecific studies of *Ae. tauschii* and can be analyzed by genealogical approaches.

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Introduction

Chloroplast DNA (cpDNA) variation has been used for evolutionary, ecological, and phylogeographic plant studies. Chloroplast DNA has a conservative nucleotide substitution rate, which facilitates comparison of variations in a wide range of plant taxa. Uniparentally inherited, the effectively non-recombining genome of the chloroplast helps simplify theories of phylogenetic inference. Comparative analyses of cpDNA variations have complemented morphological studies and provided novel phylogenetic insights, especially above the species level (e.g., Tsunewaki 1993; Provan et al. 2004).

The recent development of hypervariable chloroplast microsatellites provides a new avenue for the use of cpDNA variation as a tool to study intraspecific processes. Because the chloroplast genome is effectively haploid, population differentiation can be accelerated in it by genetic drift due to the small effective population size relative to nuclear autosomes. Hypervariable chloroplast microsatellites, coupled with accelerated population differentiation of the chloroplast genome, therefore provide useful measures for comparing intraspecific taxa and populations (Provan et al. 2001).

Chloroplast microsatellite variation is often assayed by gel electrophoresis of PCR products amplified by means of primers specific to the 5′ and 3′ flanking regions of stretches of the microsatellite repeat. Size homoplasy, however, is reported to occur in chloroplast microsatellites through recurrent generation of alleles of identical size (Doyle et al. 1998). Several types of mutation appear to be responsible for size homoplasy, e.g., gain/loss mutations of microsatellite repeat units and base substitutions and length mutations in the flanking regions (Hale et al. 2004). Size homoplasy makes it difficult to determine unambiguously whether

alleles are identical due to descent or are identical in state, thereby limiting the use of chloroplast microsatellites for phylogenetic inference.

The presence of base substitutions and length mutations in flanking regions indicates that chloroplast microsatellites, as is often the case with their nuclear counterparts (Colson and Goldstein 1999; Matsuoka et al. 2002), undergo complex mutations. Detailed analyses of mutation patterns therefore should help clarify concerns about size homoplasy in the use of chloroplast microsatellites for phylogeny reconstruction. Furthermore, polymorphisms associated with base substitutions and length mutations in the flanking regions, if identified explicitly, can serve as phylogenetic markers relatively free of recurrent mutations. To fully obtain the evolutionary information held in chloroplast microsatellite loci, it is necessary to understand the nature of mutations that underlie observed variations.

We report nucleotide sequence variations in ten microsatellite loci of the chloroplast genome of the wild grass species, *Aegilops tauschii* Coss. *Ae. tauschii*, the D-genome progenitor of bread wheat [(*Triticum aestivum*) Kihara 1944; McFadden and Sears 1944], is distributed from Syria to China, the center of distribution being in the southern coastal region of the Caspian Sea and Azerbaijan (Slageren 1994). Sixty-three accessions of *Ae. tauschii* from the collection of the 1955 Kyoto University Scientific Expedition to the Karakoram and Hindukush (Kihara et al. 1965) were selected for analyses. These accessions, which cover the central part of the *Ae. tauschii* distribution (Fig. 1), are suitable materials for investigating nucleotide sequence variation in the chloroplast microsatellites of this species. Our analyses show that (1) such different mutation types, as base substitutions and length mutations, occur in the flanking regions of the chloroplast microsatellites of *Ae. tauschii*; (2) biallelic polymorphisms associated with mutations in flanking regions can be used to investigate intraspecific patterns of monophyletic lineage divergence; and (3) highly variable multiallelic microsatellites can be used to compare intralineage diversity. Our findings show that the chloroplast genome harbors variations that provide evolutionary information for intraspecific studies of *Ae. tauschii*.

Materials and methods

Plant materials

Sixty-three accessions of *Ae. tauschii* Coss. (syn. *Aegilops squarrosa* L.), collected by the Kyoto University Scientific Expedition to the Karakoram and Hindukush, were used (see Kihara et al. 1965), 26 accessions from Afghanistan and Pakistan, 30 from the Caspian region of Iran, and seven from the inland region of Iran (Fig. 1). These accessions are maintained at the Plant Germ-plasm Institute of Kyoto University (Kawahara 1997). Locality data for the plant materials can be found in the Electronic Supplementary Material, Table 1.

Chloroplast microsatellite analysis

Total DNA was extracted from the young leaves of individual plants by the CTAB method (Doebley and Stec 1991; Saghai-Maroof et al. 1984). Chloroplast microsatellites were amplified by PCR via primers originally designed by Ishii et al. (2001) for bread wheat chloroplast microsatellites. The PCR reaction mixture consisted of a 50-ng-total DNA template, 5 nmol each dNTP, 10 pmol each primer, 2 μl of 10X buffer, 0.1 U *Ex Taq* polymerase (TaKaRa), and distilled water to 50 μl. Amplification was done in a Model 9700 (Applied Biosystems) device for a total of 25 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 0.5 min. PCR products were purified with an ExoSAP-IT kit (Amersham Pharmacia Biotech), then sequenced directly with one of the PCR primers and a BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Sequences were

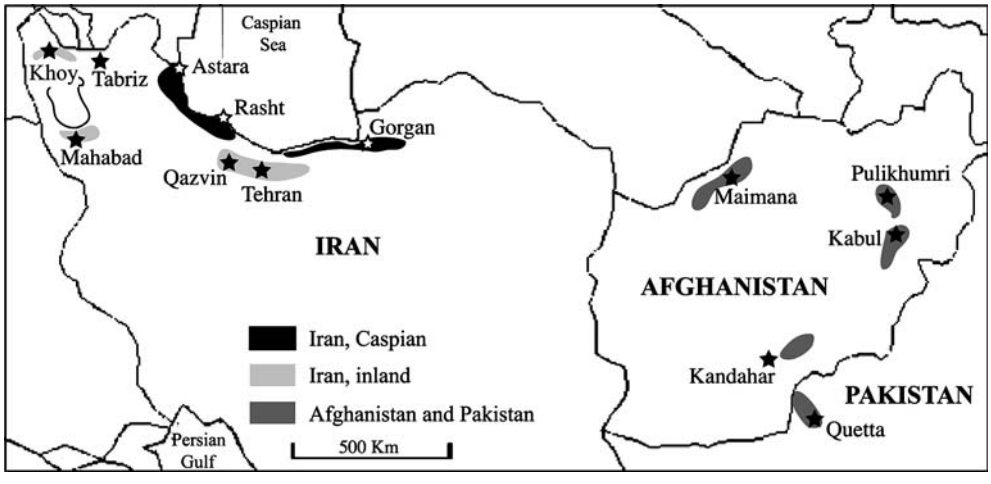

Fig. 1 Distribution of the *Aegilops tauschii* accessions analyzed. Sample areas are *shaded* according to the geographic key in the figure

analyzed with an ABI automated sequencer (Applied Biosystems). Clustal W, version 1.8 (Thompson et al. 1994), was used for nucleotide sequence alignment, and Network software version 4.101 (available at http://www.fluxus-engineering.com; Bandelt et al. 1995), for reduced median network construction.

Results

Chloroplast microsatellite sequencing

The ten PCR primer sets, originally designed for bread wheat chloroplast microsatellites (Ishii et al. 2001), were successfully used for the 63 accessions of *Ae. tauschii*. A single major PCR product was obtained in each PCR reaction (data not shown), indicative that the primer sequences were conserved between *Ae. tauschii* and bread wheat. In total, 630 chloroplast DNA fragments were sequenced. The microsatellite repeat lengths of those loci were short (8–15 mononucleotide repeats), which enabled unambiguous determination of the numbers of microsatellite repeats by single-strand sequencing. In a few cases, both-strand sequencing was done for confirmation. Except for the WCt10 locus, all had mutations other than a microsatellite repeat number change were sequenced for both strands.

Mutations in chloroplast microsatellite loci

Sequence analysis of the ten chloroplast microsatellite loci revealed mutations other than microsatellite repeat number change in four (WCt6, WCt10, WCt17–WCt18, and WCt24, Table 1). In total, eight non-microsatellite mutations were present in the flanking regions of the microsatellite repeats. The WCt6 locus had an additional microsatellite (WCt6a) in the flanking region of the original microsatellite (WCt6b), which first had been identified in bread wheat (Ishii et al. 2001). A novel minisatellite with a 20-bp repeat unit (WCt24h1) was present in the flanking regions of the WCt24 locus. In addition, a mutation interrupting the stretch of the microsatellite repeat itself was present in the WCt24 locus.

Mutation other than a microsatellite repeat number change was relatively rare. Base substitutions (SNP and LBS, see Table 1) were present in as many as 5 of 63 accessions in most cases (7.9%), whereas there was a G-to-T change at the WCt6d site in 19 accessions (30.2%). Minisatellite repeat number change (WCt24h1) occurred in 16 accessions (25.4%). Even though no mutation other than microsatellite repeat number change was present in six loci (WCt1, WCt4, WCt5, WCt8, WCt12, and WCt19), our findings indicate that the chloroplast microsatellites of *Ae. tauschii* have complex mutation patterns.

Table 1 Chloroplast microsatellite loci analyzed and mutations found

Locus[a]	Mutation site[b]	Position[c]	Mutation type[d]	Allelic state[e]	Frequency[f]	Note[g]
WCt1	**WCt1a**	NA	SSR	M	NA	$(A)_{9–11}$
WCt4	**WCt4a**	NA	SSR	B	19/63	$(T)_{9–10}$
WCt5	**WCt5a**	NA	SSR	M	NA	$(A)_{10–15}$
WCt6	WCt6a	31	SSR	M	NA	$(T)_{8–10}$
	WCt6b	NA	SSR	M	NA	$(C)_{8–10}$
	WCt6c	109	SNP	B	1/63	G to T
	WCt6d	113	SNP	B	19/63	G to T
WCt8	**WCt8a**	NA	SSR	NA	NA	$(T)_8$
WCt10	**WCt10a**	NA	SSR	M	NA	$(A)_{9–12}$
	WCt10c1	97	SNP	B	1/63	T to G
WCt12	**WCt12a**	NA	SSR	M	NA	$(T)_{10–12}$
WCt17–WCt18	**WCt17a**	NA	SSR	M	NA	$(T)_{9–12}$
	WCt1718b	56	LBS	B	3/63	TTC to AAA
	WCt1718c	345	SNP	B	1/63	G to A
	WCt18a	NA	SSR	NA	NA	$(A)_9$
WCt19	**WCt19a**	NA	SSR	NA	NA	$(A)_8$
WCt24	**WCt24a**	NA	SSR/IN	M	NA	$(A)_{8–12}/(A)_5T(A)_5$
	WCt24d1	34	SNP	B	5/63	C to T
	WCt24h1	31	VNTR	B	16/63	$(CTTCGTTACCTAGTTATTTT)_{1–2}$
	WCt24j1	112	SNP	B	1/63	G to A

[a]Locus names are according to Ishii et al. (2001). The WCt17–WCt18 locus was amplified using WCt17 forward and WCt18 reverse primers

[b]Microsatellites originally identified by Ishii et al. (2001) are shown in *boldface*

[c]Positions are based on the aligned sequences, counted from the nucleotide next to the 3′ end of the forward primer, and shown only for the newly found mutation sites. *NA* Non-applicable

[d]Base substitution, interruption in microsatellite repeats, linked base substitution (i.e., small block substitution of nucleotides), microsatellite mutation, and minisatellite (i.e., hypervariable arrays with 10–50 repeat units) mutation, respectively, are denoted by *SNP*, *IN*, *LBS*, *SSR*, and *VNTR*, respectively

[e]Biallelic and multiallelic, respectively, are denoted by *B* and *M*

[f]Frequencies of the minor allele are give for biallelic sites. *NA* Non-applicable

[g]Alleles found or estimated mutation directions are given for each mutation site

Table 2 Haplogroups defined by biallelic polymorphisms

Haplogroup	No. of individuals	Biallelic site name								
		WCt4a	WCt6c	WCt6d	WCt10c1	WCt1718b	WCt1718c	WCt24d1	WCt24h1	WCt24j1
HG1	1	9	G	G	A	TTC	G	C	1	A
HG2	19	9	G	G	A	TTC	G	C	1	G
HG3	4	9	G	G	A	TTC	G	T	1	G
HG4	1	9	G	G	C	TTC	G	T	1	G
HG5	3	10	G	G	A	TTC	G	C	1	G
HG6	1	10	G	G	A	TTC	A	C	2	G
HG7	14	10	G	G	A	TTC	G	C	2	G
HG8	1	10	T	G	A	TTC	G	C	2	G
HG9	3	9	G	T	A	AAA	G	C	1	G
HG10	16	9	G	T	A	TTC	G	C	1	G

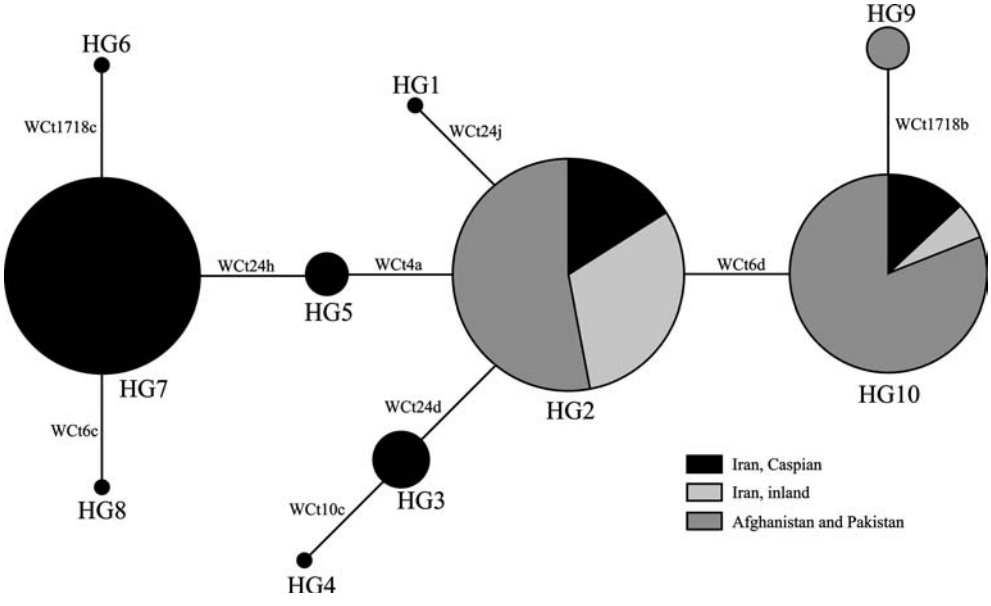

Fig. 2 Reduced-median network for the chloroplast DNA haplogroups of *Ae. tauschii* constructed using biallelic polymorphisms. The *circles* represent haplogroups with diameters proportional to the number of accessions. *Shading* shows geographic origin proportions, according to the geographic key. Mutations that define haplogroups are shown on *branches*

Evolutionary informative biallelic polymorphism

Sequence analysis of the chloroplast microsatellites of *Ae. tauschii* showed two types of segregating sites, biallelic (sites with two alleles) and multiallelic (sites with more than two alleles) (Table 1). In most cases, the biallelic sites are the result of mutations other than microsatellite repeat number change (exception: WCt4a, Table 1). Because such mutations (especially base substitutions) are considered to occur much less often than microsatellite repeat number changes, they can serve as phylogenetic markers to define intraspecific monophyletic lineages of non-recombining chloroplast genomes (Hurles and Jobling 2001).

To assess the usefulness of biallelic cpDNA polymorphisms in evolutionary studies of *Ae. tauschii*, we defined ten cpDNA haplogroups from the data on biallelic sites (Table 2), constructed a reduced median network, and examined the geographic distribution patterns of the haplogroups (Fig. 2). Each haplogroup consisted of haplotypes defined by a unique set of alleles at all the mutation sites, including multiallelic ones (see Electronic Supplementary Material, Table 1). Three major haplogroups were distinguished: HG2, HG7, and HG10 (Table 2; Fig. 2). Geographically, HG7 was restricted to the Caspian region of Iran, and HG10 was relatively rare outside of Afghanistan and Pakistan, whereas HG2 was distributed widely in the eastern (Afghanistan and Pakistan) and western (Iran) parts of the entire distribution. This marked difference in geographic distribution suggests that each haplogroup represents an intraspecific lineage that underwent a unique pattern of divergence and dispersal during *Ae. tauschii* evolution. Furthermore, by viewing the network regionally, the Caspian region of Iran obviously was the center of haplogroup diversity, harboring all but one haplogroup. Outside of that region, many fewer haplogroups were found, two (HG2 and HG10) for the inland region of Iran and three (HG2, HG9, and HG10) for Afghanistan and Pakistan (Fig. 2). These findings are consistent with those of a previous study showing the center of *Ae. tauschii*'s genetic diversity to be in the southern coastal region of the Caspian sea and Transcaucasia (Dvorak et al. 1998). In all, these findings indi-

Table 3 Genetic diversity measures for the three major chloroplast DNA haplogroups based on multiallelic polymorphisms

Genetic diversity measures	Haplogroup		
	HG2 ($n=19$)	HG7 ($n=14$)	HG10 ($n=16$)
No. of variable sites	8	4	5
No. of haplotypes	10	6	8
Haplotype diversity index[a]	0.81	0.74	0.83
No. of alleles per site	2.88 ± 0.93	1.63 ± 0.70	2.13 ± 1.05
Mean allele diversity index	0.36 ± 0.15	0.14 ± 0.19	0.28 ± 0.24

[a]Calculated as $1\text{-}\Sigma p_i$, where p_i is the frequency of the ith haplotype

cate that the geographic distribution patterns of the ten haplogroups reflect the species' history of divergence and dispersal. Biallelic cpDNA polymorphisms therefore can be used for intraspecific studies of *Ae. tauschii*.

Intralineage diversity

Because biallelic sites are considered relatively free of recurrent mutations, each of the ten haplogroups represents a monophyletic lineage. A comparison of genetic diversity between these monophyletic lineages is of evolutionary interest. This may be done with genetic markers that vary within lineages. In our case, multiallelic sites (Table 1) could be used for this purpose.

To test whether multiallelic sites can be used to distinguish haplogroup genetic diversity, we examined genetic diversity measures for the three major haplogroups: HG2, HG7, and HG10 (Table 3). These haplogroups were chosen because of their large and nearly equal sample sizes (Table 2). Eight multiallelic sites (WCt1a, WCt5a, WCt6a, WCt6b, WCt10a, WCt12a, WCt17a, and WCt24a) were analyzed. Genetic diversity measures tended to be small in HG7 relative to HG2 and HG10 (Table 3), to which the small HG7 sample size may have contributed. Two genetic diversity measures that are less sensitive to sample size difference (the haplotype diversity and mean allele diversity indices), however, were reduced in HG7, indicative that it is genetically less diverse than the other haplogroups. These findings show that multiallelic sites of the chloroplast genome can be used to compare the genetic diversity of haplogroups.

Discussion

Complex mutations at chloroplast microsatellites

Nuclear microsatellites have complex mutation patterns. For example, microsatellite repeat numbers may change asymmetrically, some tending to increase, others to decrease (Huang et al. 2002). Interruptions of the stretches of microsatellite repeats may stabilize microsatellite loci (Kruglyak et al. 1998); moreover, a form of allele-size constraint seems to prevent microsatellites from having a large number of repeats (see Goldstein and Pollock 1997). In addition, mutations in the flanking regions of microsatellites (such as indels and base substitutions) are common in plant (Matsuoka et al. 2002) and animal (Colson and Goldstein 1999) nuclear microsatellite loci.

We analyzed the nucleotide sequences of ten chloroplast microsatellites of *Ae. tauschii* and found that several types of mutations occur in those loci (Table 1). Base substitutions and length mutations were present in the flanking regions of four loci: WCt6, WCt10, WCt17–WCt18, and WCt24. In addition, a mutation that interrupts the microsatellite repeat stretch was present in the WCt24 locus. These findings indicate that, as is often the case with the nuclear microsatellites, the chloroplast microsatellites of *Ae. tauschii* have complex mutation patterns.

Because no length mutation was found in the flanking regions of eight loci, the percentage of *Ae. tauschii* chloroplast microsatellites having size variations due to length mutation in the flanking regions should not be as high as the values reported for maize (87%) and fruit fly (60%) nuclear microsatellites (Colson and Goldstein 1999; Matsuoka et al. 2002). The conventional gel assay therefore provides a simple method for analyzing chloroplast microsatellite repeat number variation. In fact, a gel assay is much more cost-efficient than sequencing, especially when dealing with a large number of samples. The number of base substitutions present in the ten chloroplast microsatellite loci (six SNPs and one LBS, see Table 1), however, highlights the need for sequencing in order to obtain all the evolutionary information present in the chloroplast microsatellite loci. Furthermore, nucleotide sequence analyses should help us understand better the mutation process in chloroplast microsatellite loci and to develop phylogenetic methods based on our knowledge of the genetic mechanism that generates variation.

Comparison of chloroplast microsatellite repeat variations

To obtain insight into the chloroplast microsatellite evolution in *Aegilops* and *Triticum*, *Ae. tauschii* and *Ae. speltoides*, and the tetraploid *Triticum* wheats, microsatellite repeat variations were compared (Table 4). *Aegilops* and *Triticum* are congenic, and *Ae. speltoides* and the tetraploid *Triticum* wheats have closely related plasmons (type B, G, and S), whereas *Ae. tauschii* has the type D plasmon (Tsunewaki 1993). Accessions of *Ae. speltoides* and the tetraploid *Triticum* wheats used in the comparison had been analyzed previously for the loci used in this study (Ishii et al. 2001). To quantify variability, a diversity index was calculated for each locus using $1-\sum p_i$, where p_i is the frequency of the ith microsatellite allele (i.e., "gene diversity," Nei 1973).

Similar mean values were obtained for the number of alleles per locus (2.909 for *Ae. tauschii*, 2.818 for *Ae.*

Table 4 Comparison of chloroplast microsatellite variations among *Ae. tauschii* and the *Ae. speltoides* and tetraploid *Triticum* wheats

Microsatellite	*Ae. tauschii*			*Ae. speltoides* and the 4*xTriticum* wheats[b]		
	No. of individuals[a]	No. of alleles	Diversity index (*H*)	No. of individuals	No. of alleles	Diversity index (*H*)
WCt1a	63	3	0.148	29	2	0.485
WCt4a	63	2	0.421	29	4	0.535
WCt5a	63	5	0.695	29	4	0.516
WCt6b	63	3	0.386	29	3	0.473
WCt8a	63	1	0	29	1	0
WCt10a	63	4	0.660	29	2	0.067
WCt12a	63	3	0.610	29	3	0.595
WCt17a	63	4	0.742	29	3	0.471
WCt18a	63	1	0	29	1	0
WCt19a	63	1	0	29	3	0.625
WCt24a	62	5	0.468	29	5	0.644
Mean		2.909	0.375		2.818	0.401

[a]One accession with an interruption in the WCt24 microsatellite repeat was excluded from the calculations
[b]Calculated from data for *Ae. speltoides* (one accession) and the tetraploid *Triticum* wheats (*T. dicoccoides*, *T. dicoccum*, *T. durum*, *T. araraticum*, and *T. timopheevi*, 28 accessions in total) (Ishii et al. 2001)

speltoides and the tetraploid *Triticum* wheats), whereas the mean diversity index was slightly higher in *Ae. speltoides* and the tetraploid *Triticum* wheats (0.401) than in *Ae. tauschii* (0.375). Taking into account the small number of accessions analyzed for *Ae. speltoides* and the tetraploid *Triticum* wheats, these results suggest that chloroplast microsatellites vary less in *Ae. tauschii* than in *Ae. speltoides* and tetraploid *Triticum* wheats. Of the three microsatellites monomorphic in *Ae. tauschii*, two (WCt8a and WCt18a) also were monomorphic in *Ae. speltoides* and the tetraploid *Triticum* wheats, and the other (WCt19a) was polymorphic. Pearson's correlation coefficients were positive for both the numbers of alleles per locus (0.624) and the diversity indices (0.327) but significant only for the number of alleles per locus ($P = 0.040$). These results must be viewed with caution, because the data set for *Ae. speltoides* and the tetraploid *Triticum* wheats was obtained by gel assay (Ishii et al. 2001) and may not adequately reflect the variability resulting from length mutations in flanking regions.

Genealogical use of cpDNA variation in intraspecific studies of *Ae. tauschii*

The human Y chromosome is haploid, non-recombining, and uniparentally inherited. These characteristics are shared by chloroplast genomes of many plant species. In humans, genealogical approaches that combine data from biallelic and multiallelic sites of the Y chromosome hierarchically have been used successfully to construct haplogroup networks, to analyze geographic distribution patterns of haplotypes, to estimate the ages of intraspecific lineages, and to discriminate between closely related populations (Bosch et al. 1999; de Knijff et al. 1997). This approach also may be applicable to cpDNA variation in plants.

We here showed that the chloroplast genome of *Ae. tauschii* has two types of mutation sites, biallelic and multiallelic, in the flanking regions of its microsatellite repeats. We used biallelic polymorphisms to construct a reduced median haplogroup network that represents the evolutionary pathway marked by unique mutation events during the evolution of *Ae. tauschii* (Fig. 2). Phylogeographic insights provided by the haplogroup network were consistent with those of previous genetic *Ae. tauschii* studies. Furthermore, analyses of intralineage diversity showed that data on multiallelic sites could be used to compare genetic diversity between monophyletic lineages. This result indicates that multiallelic sites have the potential for phylogenetic use, because, as shown for human Y haplogroups (Bosch et al. 1999; de Knijff et al. 1997), comparative analyses of genetic diversity can provide insights into the formation, demography, and diversification of intraspecific lineages. These findings clearly indicate that the genealogical approaches used in human Y chromosome studies are applicable to the analysis of cpDNA variation in *Ae. tauschii*. Because the power of genealogical analyses relies heavily on the number of biallelic sites that define monophyletic lineages, it is desirable to find more of these sites (especially those associated with base substitutions) by exploring other regions of the *Ae. tauschii* chloroplast genome.

Ae. tauschii is morphologically, ecologically, and genetically diverse (Slageren 1994). Because *Ae. tauschii* is the D-genome progenitor of bread wheat, understanding its genetic population structure is fundamental to the full use of the vast *Ae. tauschii* germplasm for wheat breeding. Phylogenies based on chloroplast and nuclear DNA variations are often incongruent (Doyle et al. 1999; Nishimoto et al. 2003; Sasanuma et al. 2004). Comparative analyses of incongruent phylogenies may help infer the impact of hybridization and lineage sorting on the species population structure (Wendel and Doyle 1998). Genealogical analyses of cpDNA variations of *Ae. tauschii* therefore provide an alternative approach that has the potential to complement previous

work done with nuclear markers (Dvorak et al. 1998; Guyomarc'h et al. 2002; Lelley et al. 2000; Pestsova et al. 2000). In general, a broad sample set covering the entire species range is required to address questions regarding the history of the formation, dispersal, and diversification of a species. Large-scale genealogical analyses of cpDNA variations may provide a means of better understanding the morphological, ecological, and genetic diversity of *Ae. tauschii*.

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