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ORIGINAL PAPER

Natural variation for fertile triploid F_1 hybrid formation in allohexaploid wheat speciation

Yoshihiro Matsuoka · Shigeo Takumi · Taihachi Kawahara

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Abstract The tempo, mode, and geography of allopolyploid speciation are influenced by natural variation in the ability of parental species to express postzygotic reproductive phenotypes that affect hybrid fertility. To shed light on the impact of such natural variations, we used allohexaploid *Triticum aestivum* wheats' evolution as a model and analyzed the geographic and phylogenetic distributions of *Aegilops tauschii* (diploid progenitor) accessions involved in the expression of abnormality and fertility in triploid F_1 hybrids with *Triticum turgidum* (tetraploid progenitor). Artificial-cross experiments and chloroplast-DNA-based evolutionary analyses showed that hybrid-abnormality-causing accessions had limited geographic and phylogenetic distributions, indicative that postzygotic hybridization barriers are underdeveloped between these species. In contrast, accessions that are involved with fertile triploid F_1 hybrid formation have wide geographic and phylogenetic distributions, indicative of a deep evolutionary origin. Wide-spread hybrid-fertilizing accessions support the theory that *T. aestivum* speciation occurred at multiple sites within the species range of *Ae. tauschii*, in which existing conditions enabled natural hybridization with *T. turgidum*. Implications of our findings on how natural variation in the ability of *Ae. tauschii* to express those postzygotic reproductive phenotypes diversified and contributed to the speciation of *T. aestivum* are discussed.

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Y. Matsuoka (✉)
Fukui Prefectural University, Matsuoka,
Eiheiji, Yoshida, Fukui 910-1195, Japan
e-mail: matsuoka@fpu.ac.jp

S. Takumi
Department of Biological and Environmental Science,
Faculty of Agriculture, and Graduate School of Science
and Technology, Kobe University, Kobe,
Hyogo 657-8501, Japan

T. Kawahara
Graduate School of Agriculture, Kyoto University,
Muko, Kyoto 617-0001, Japan

Introduction

Polyploid speciation is one of the most important evolutionary processes in plants and animals. Forty-seven to seventy percent of flowering plant species are estimated to be descendants of polyploid progenitors (Masterson 1994). A widespread class of polyploids consists of allopolyploids derived from hybrids between different species. In nature, allopolyploid species form through a complex process that involves species crosses, fertile hybrid development, chromosome doubling, and diploidization. Despite recent progress in understanding the molecular mechanisms that stabilize newly formed hybrid genomes (Soltis et al. 2003), our knowledge of the genetic and ecological factors that affect the early stages of allopolyploid speciation is limited.

Allopolyploid speciation is prevented or promoted by postzygotic reproductive phenotypes that affect hybrid fertility. Examples of postzygotic phenotypes that negatively affect this fertility are abnormalities such as lethality, weakness, and sterility. Abnormal phenotypes lower hybrid fertility by impairing viability or reproductive potential. Hybrid abnormality is common in plants and animals and constitutes a major class of postzygotic barriers for reproductive isolation (Coyne and Orr 2004). One postzygotic

phenotype that positively affects hybrid fertility is 2*n* gamete production. This produces functional gametes with somatic chromosome numbers (i.e. 2*n* gametes) and brings about chromosome doubling through the union of male and female 2*n* gametes. As a result, interspecific F_1 hybrids are capable of producing F_2 hybrids that have cytologically and genetically stable amphidiploid genomes.

Postzygotic reproductive phenotypes often are controlled by genetic factors transmitted from parental species or strains (Xu and Joppa 1995; Presgraves et al. 2003). Because fertile hybrid formation is a key step in allopolyploid speciation, knowledge about when and how often the genetic ability to express postzygotic reproductive phenotypes arose in the course of parental species' evolution is critical for a better understanding of that speciation. One possible approach is to perform a systematic cross study of a broad set of parental species populations and compare hybrid phenotypes and fertilities. This should enable us to detect and analyze intraspecific variation in the ability to express postzygotic reproductive phenotypes in terms of the geography and phylogeny of parental species and, ultimately, to assess the impact of such postzygotic phenotypes on allopolyploid formation.

Progenitors of the hexaploid common wheats *Triticum turgidum* L. (AABB genome) and *Aegilops tauschii* Coss. (DD genome) provide a suitable species system for such a study. Allohexaploid *Triticum aestivum* wheats (*T. aestivum* L. ssp. *aestivum*, ssp. *compactum*, ssp. *macha*, ssp. *spelta*, and ssp. *sphaerococcum*, AABBDD genome) are considered to be derived from a hybrid cross between a cultivated form of *T. turgidum* (female progenitor) and a wild species of *Ae. tauschii* (male progenitor) which had wide genetic and phenotypic amplitudes and natural populations that adapted to diverse ecogeographic conditions in central Eurasia (Kihara 1944; McFadden and Sears 1944; Van Slageren 1994; Tanaka and Tsujimoto 1991; Zohary and Hopf 2000). By making artificial crosses between *T. turgidum* and *Ae. tauschii*, the natural process of *T. aestivum*'s speciation of approximately 8,000 years ago can be reproduced without using chemicals or embryo rescue techniques (Kihara and Lilienfeld 1949). Furthermore, triploid F_1 hybrids between *T. turgidum* and *Ae. tauschii* (ABD genome) display diverse postzygotic reproductive phenotypes ranging from lethality to high-rate seedsetting (>50%) (Nishikawa 1960; Matsuoka and Nasuda 2004).

We report results of a systematic cross study that used a cultivar of *T. turgidum* as the female tester in order to scan an array of *Ae. tauschii* accessions for natural variation in the ability to express postzygotic reproductive phenotypes. Hybrid abnormality, selfed-seedset rate, and pollen fertility were the foci, important phenotypes for the production of hexaploid offspring. Our goals were (1) to picture the geographic and phylogenetic distributions of the *Ae. tauschii* accessions involved in the expression of postzygotic reproductive phenotypes in triploid F_1 hybrids with *T. turgidum*, (2) to infer the intraspecific process generating variation in *Ae. tauschii*'s ability to express postzygotic reproductive phenotypes, and (3) to obtain insight into the impact of diverse postzygotic reproductive phenotypes on the allopolyploid speciation of *T. aestivum*. We show that hybrid-abnormality-causing and hybrid-fertilizing accessions have contrasting geographic and phylogeographic distribution patterns and discuss how *Ae. tauschii*'s variation in the ability to express those postzygotic reproductive phenotypes diversified and contributed to the allopolyploid speciation of *T. aestivum*.

Materials and methods

Plant materials

Parental materials were the durum wheat cultivar Langdon (*T. turgidum* ssp. *durum* cv. "Langdon", hereafter Ldn) and 74 accessions of *Ae. tauschii* Coss. (syn. *Ae. squarrossa* L.) (Table S1). When geographical coordinates of the sampling sites were not available in the original passport data, we estimated latitude and longitude by means of Kashmir 3D software (available at http://www.kashmir3d.com/) on scanned paper maps (scales 1:4,000,000–1:1,000,000) based on the locality information provided by the seed banks.

Production of hybrids

Seeds were sown in December, and plants grown individually in pots in a greenhouse at Fukui Prefectural University. The greenhouse was heated weakly during the first 3–4 weeks to enhance early development but thereafter was unheated. To produce F_1 hybrids, Ldn spikes were fully emasculated and pollinated by hand with an *Ae. tauschii* accession as the pollen parent. No gibberellic acid solution was applied to the embryos after pollination. After harvest, crossability was calculated as the number of seeds obtained/number of florets pollinated. Well-developed F_1 seeds were germinated in Petri dishes at 23°C then transplanted. Neither colchicine nor similar chemicals were applied to the F_1 plants. F_1 plant hybridity was confirmed by the use of multiple morphological markers coleoptile color, waxiness, tough glume, and lax-internodes of spikes.

Measures of hybrid fertility

The fertility of Ldn-*Ae. tauschii* triploid F_1 hybrids was assayed by the measures of selfed-seedset rates and pollen fertility (Matsuoka and Nasuda 2004). The selfed-seedset

rate was calculated as the number of seedsets/number of well-developed florets × 100. For selfing, spikes on well-developed tillers were bagged before flowering. Fertility of pollen grains sampled from mature anthers at flowering was measured. Grains were stained in 2% aceto-carmine solution with glycerin. At least 1000 grains per plant were observed. Pollen fertility was calculated as the number of normal pollen grains/total pollen count × 100. F_2 hybrid plants were grown in a field at Kobe University. Their selfed-seedset rates were measured as above.

Chloroplast DNA haplotype analysis

Each *Ae. tauschii* accession was analyzed for chloroplast DNA (cpDNA) variation at the biallelic base-change and minisatellite sites in the flanking regions of chloroplast microsatellite loci. In this study, new cpDNA haplotype data were obtained for 46 accessions, and data for 28 accessions were taken from our previous study (Matsuoka et al. 2005). Total DNA was extracted from the young leaves of individual plants by the cTAB method (Saghai-Maroof et al. 1984; Doebley and Stec 1991). Chloroplast DNA fragments 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 *n*mol each of dNTPs, 10 *p*mol of each primer, 5 µl of 10 × buffer, 0.1 unit of *Ex Taq*™ polymerase (Takara), and distilled water to 50 µl. Amplification was done in a Model 9700 (Applied Biosystems) device for 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 in a BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Sequences were analyzed in an ABI automated sequencer (Applied Biosystems). Clustal W ver. 1.8 (Thompson et al. 1994) was used for nucleotide sequence alignment, and Network software ver. 4.112 (available at http://www.fluxus-engineering.com) (Bandelt et al. 1995) for reduced median network construction. Other statistical calculations were done with JMP software ver. 5.1.2 (SAS Institute).

Results

Totally, 74 accessions of *Ae. tauschii* that covered the entire species' natural distribution range were crossed with Ldn (Fig. 1a). For each combination, 11–190 Ldn plant florets were crossed. Crossability ranged from 0 to 0.83 (average 0.29). Small well-developed F_1 hybrid seeds were obtained from 66 cross combinations (89.2%) (Table S1). To evaluate fertility, 1–14 F_1 hybrid seeds were sown from each successful combination. F_1 hybrids from 25 (37.9%) successful cross combinations showed abnormal phenotypes, whereas those from 41 (62.1%) successful cross combinations grew normally. Germination rates varied from 0 to 1.00 (average 0.77) depending on the cross combination (Table S2).

Hybrid abnormality

We classified hybrid abnormality phenotypes in three categories: germination failure, severe dwarfness, and necrotic dysgenesis (Fig. 2, Table S2). Germination failure (zero germination rate) occurred in F_1 hybrids from five cross combinations. In crosses that produced germination-failure hybrids, except when KU20-7 was the male parent (0.50), crossability was relatively low (0.06–0.21) (Table S1). F_1 hybrids from five cross combinations had the severe dwarfness phenotype (Fig. 2a). This phenotype resembled the Type1–dwarf of bread wheat (Hermsen 1967). Severe-dwarfness plants ceased growing in the early seedling stage. Under our conditions, they never set spikes, but in most cases lived for about 6 months after germination. Fifteen cross combinations produced F_1 hybrids that showed such symptoms of necrotic dysgenesis as severe chlorophyll depletion in the seedling stage and incomplete leaf emergence from the leaf sheath in the tillering stage (Nishikawa 1960, 1962) (Fig. 2b and c, see d for comparison). Of them, F_1 hybrids from four cross combinations involving accessions CGN10767, IG 47196, KU-2809, and KU-2834 were fully lethal, whereas 11 cross combinations produced necrotic hybrid plants that grew to maturity.

Aegilops tauschii accessions that caused hybrid abnormality had two geographic centers within the species' natural distribution range, the Transcaucasus and Afghanistan–Pakistan border areas (Fig. 1b). Accessions that caused germination failure and necrotic dysgenesis were distributed in both centers, whereas those that caused severe dwarfness were confined to the Transcaucasus. A large number of accessions (22) from the southern-coastal Caspian region were tested (Fig. 1a). Notably, none was involved with the expression of hybrid abnormality.

Hybrid fertility

We assayed the fertility of triploid F_1 hybrids from 41 normal and 11 necrotic cross combinations by using the selfed-seedset rates (mainly for female fertility assays) and pollen fertility (for male fertility assays) as measures. All F_1 hybrid plants that grew to maturity set well-developed seeds. No fully sterile individual was found. Selfed-seedset rates (7.5–68.3%) and pollen fertility (18.8–94.4%), however, varied greatly depending on the cross combination (Table S3). As is often the case with newly formed polyploids (Ramsey and Schemske 2002), the percentage degrees

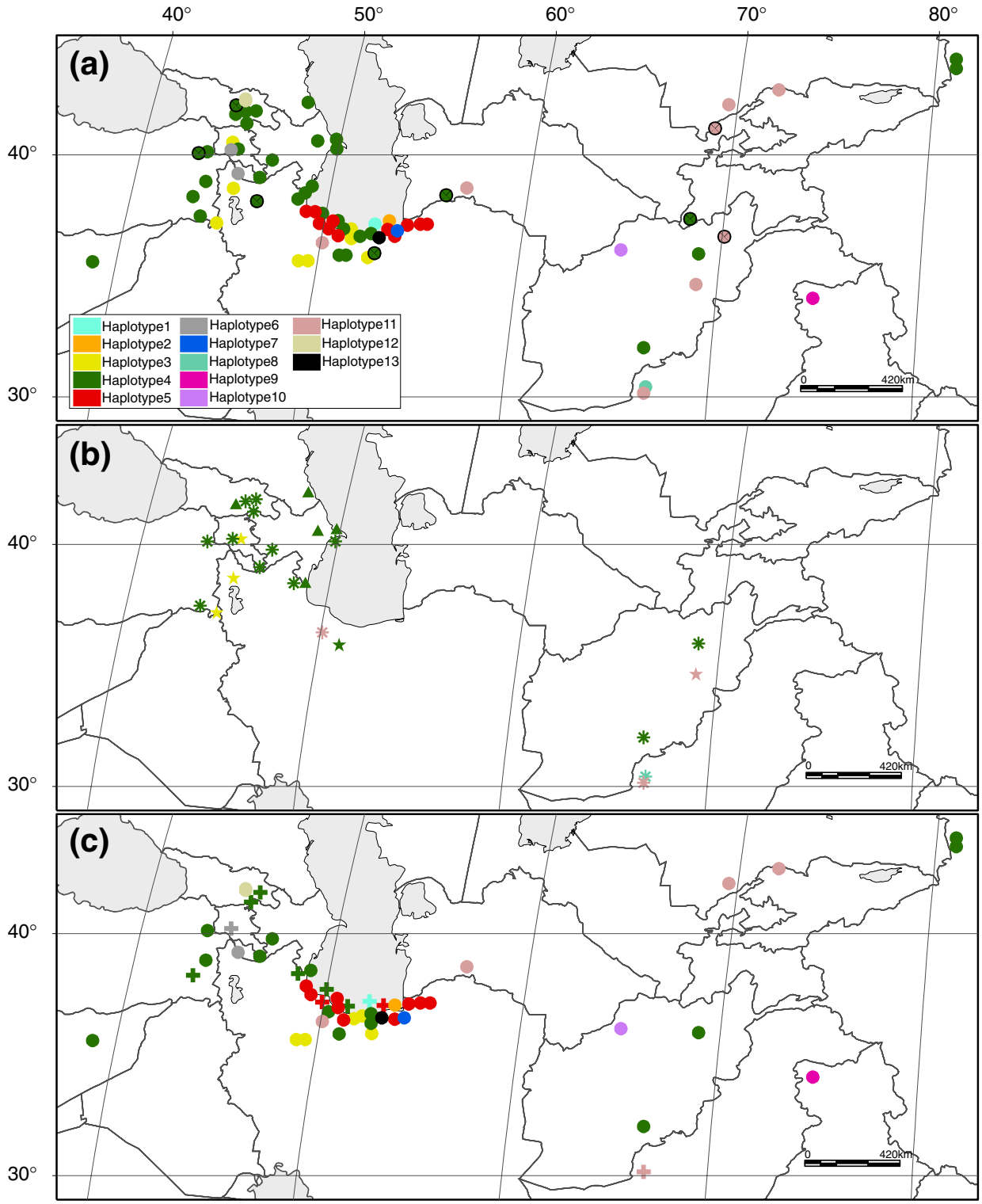

Fig. 1 Geographic distributions of *Ae. tauschii* accessions crossed with Ldn. **a** Distributions of all 74 accessions crossed with Ldn. See text for details of cpDNA haplotypes. *Color key*: common to **b** and **c**. **b** Distributions of hybrid-abnormality-causing accessions. *Stars* indicate germination-failure, *asterisks* necrotic-dysgenesis, and *triangles* severe-dwarfness accessions. **c** Distributions of hybrid-fertilizing accessions. *Thick crosses* indicate accessions involved in highly fertile cross combinations (F_1 hybrid seedset rate > 50%). Averaged rates are plotted for data obtained in 2004 and 2005

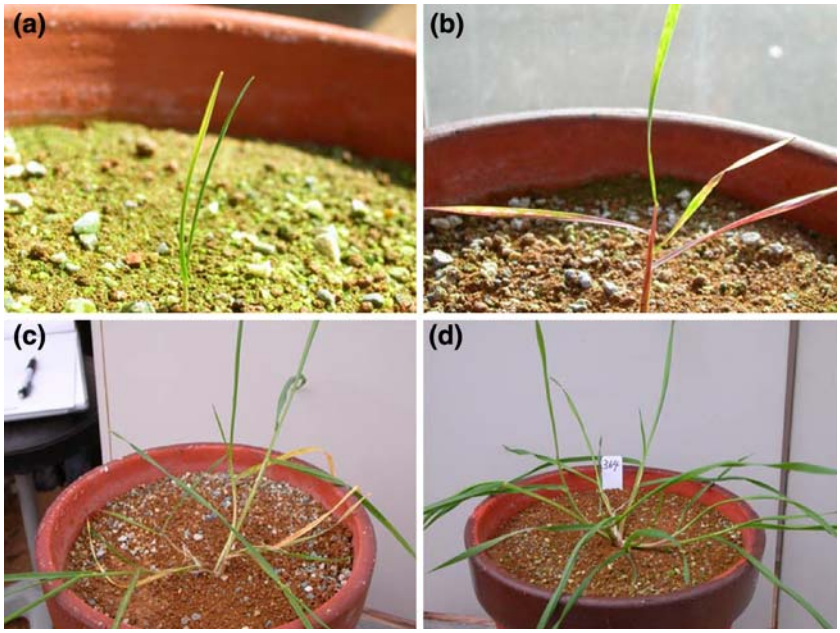

Fig. 2 Examples of phenotypes observed in Ldn-*Ae. tauschii* triploid F_1 hybrids. **a** A 30-day-old hybrid plant (*Ae. tauschii* accession AE 1037) with the severe-dwarfness phenotype (*height* about 3 cm). **b** A 52-day-old hybrid plant (*Ae. tauschii* accession PI 486274) with systemic expression of necrotic symptoms. **c** A 59-day-old necrotic dysgenesis hybrid plant (*Ae. tauschii* accession KU-2826). Note the *yellow* lower leaves and weak tillering compared to the normal plant **d**. This plant grew to maturity and showed high fertility (Table S3), whereas other plants derived from the same cross combination died young due to systemic necrotic symptoms. Pot diameter, 19 cm. **d** A 50-day-old hybrid plant (*Ae. tauschii* accession KU-2088) with the normal phenotype

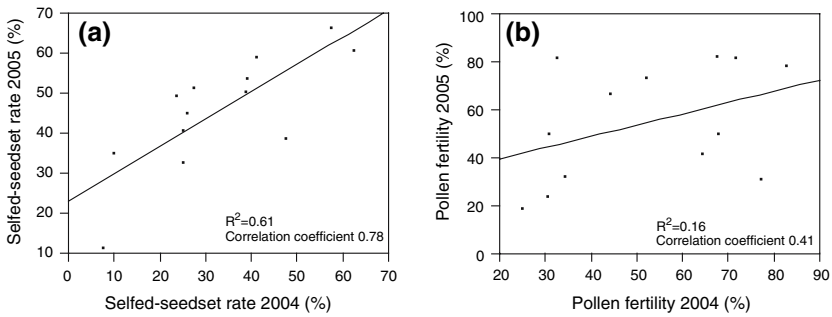

Fig. 3 Relationships between years 2004 and 2005 measures for hybrid fertility. **a** Selfed-seedset rates. **b** Pollen fertility

of pollen fertility mostly were higher than those of the selfed-seedset rates. Interestingly, some hybrids from necrotic cross combinations had high selfed-seedset rates (12.5–61.3%) and pollen fertility (62.0–94.4%). F_2 hybrids derived from the year 2003 crosses also showed high selfed-seedset rates (average 89.0%, range 58.3–97.7%) (Table S4), even though chromosome pairing often was unstable in newly synthesized wheat amphidiploids (Kihara et al. 1965). These findings show that all the *Ae. tauschii* accessions involved in the 41 normal and 11 necrotic cross combinations had the ability to fertilize the triploid F_1 hybrids with Ldn.

Hybrid selfed-seedset rates and pollen fertility may be affected by environmental factors. In our study, average selfed-seedset rates and pollen fertility were higher for F_1 hybrids grown in 2005 (average selfed-seedset rates = 43.3%, pollen fertility = 61.0%, $n = 35$) than in 2004 (average selfed-seedset rates = 33.0%, pollen fertility = 58.2%, $n = 31$) (Table S3). In fact, 13 F_1 hybrids grown in both years showed higher average selfed-seedset rates (33.2% for 2004, 45.7% for 2005, $t = 4.32$, $P = 0.0010$) and pollen fertility (52.3% for 2004, 54.7% for 2005, $t = 0.35$, $P = 0.73$) in 2005 than in 2004, evidence that environmental factors affected the yearly fluctuation. A positive correlation between the 2004 and 2005 selfed-seedset rates, however, indicated that some genetic factor(s) transmitted from the parental *Ae. tauschii* accessions affected female fertility (Fig. 3a). Pollen fertility data showed a positive but non-significant correlation (Fig. 3b), indicative that this measure of hybrid fertility might tend to fluctuate, depending on environmental factors.

Table 1 Multiple regression analyses of effects of geographic origin on selfed-seedset rates and pollen fertility of triploid Ldn-*Ae. tauschii* F_1 hybrids

Source	Parameter estimate	Standard error	*t*-statistic	*P*
A				
Latitude	0.01	0.01	0.46	0.65
Longitude	0.00	0.00	−0.35	0.73
B				
Latitude	0.01	0.01	0.97	0.34
Longitude	0.00	0.00	0.13	0.90
C				
Latitude	0.01	0.01	0.66	0.52
Longitude	0.00	0.00	−0.02	0.98
D				
Latitude	0.03	0.02	1.83	0.08
Longitude*	0.01	0.00	2.22	0.03

A, Selfed-seedset rate 2004 ($N = 30$); B, Pollen fertility 2004 ($N = 30$); C, Selfed-seedset rate 2005 ($N = 34$); D, Pollen fertility 2005 ($N = 34$). Asterisk indicates a source with significant effects ($P < 0.05$)

Aegilops tauschii accessions that fertilize the hybrids are distributed widely throughout the species' natural distribution range (Fig. 1c). A cluster of accessions involved in crosses that produced highly fertile F_1 hybrids (selfed-seedset rate > 50%) was found in the southern-coastal Caspian region and Transcaucasus (Fig. 1c). To test whether variations in selfed-seedset rates and pollen fertility are associated with latitudinal and longitudinal transitions, we performed multiple regression analyses using the latitude and longitude of *Ae. tauschii* sampling sites as the independent values and seedset rates and pollen fertility as the response values. Separate analyses were done for the 2004 and 2005 observations (Table 1). A significant association with longitudinal transition was found for 2005 pollen fertility (Table 1D). This, however, requires further testing because of the relatively low reproducibility of pollen fertility data. No significant association with latitudinal and longitudinal transitions was found for seedset rates, indicative that variation in the female fertility has neither a simple latitudinal nor longitudinal cline.

Phylogeny

To further investigate intraspecific variation in the ability of *Ae. tauschii* to express postzygotic reproductive phenotypes in triploid F_1 hybrids with Ldn, we analyzed the within-species phylogeny of *Ae. tauschii* using cpDNA variations and examined the phylogenetic distributions of hybrid-abnormality-causing and hybrid-fertilizing accessions. Because *Ae. tauschii* is a selfing species, its uniparentally-inherited chloroplast genome provides useful markers for tracing intraspecific genealogical divergence (Matsuoka et al. 2005). In our present study, 14 biallelic base-change and minisatellite sites were used to analyze the cpDNA haplotypes of 74 accessions crossed with Ldn (Table S5). Thirteen cpDNA haplotypes were identified in this set of accessions, and a star-shaped reduced-median network was obtained by phylogenetic analyses (Fig. 4). Consistent with our previous findings (Matsuoka et al. 2005), chloroplast haplotypes were diverse in western habitats (longitude < 60°E) (10 haplotypes) relative to eastern ones (longitude ≥ 60°E) (five haplotypes) (Fig. 4a). This is consistent with the center of *Ae. tauschii*'s genetic diversity being in the Caspian and Transcaucasus regions (Dvorak et al. 1998). Geographically, the large haplotype4 is at the center of the network distributed widely across the current species' natural distribution range from Syria to China, whereas the other haplotypes (except number 11) are confined either to east or west of longitude 60°E (Fig. 1a), evidence that haplotype4 represents the lineage ancestral to the majority of current *Ae. tauschii* populations that have other haplotypes.

The 25 hybrid-abnormality-causing accessions were found to have haplotypes 3, 4, 8, and 11 (Fig. 4b, Table S2). Haplotype4 was common to germination-failure, severe-dwarfness, and necrotic-dysgenesis accessions and was the solo haplotype for severe-dwarfness accessions. Furthermore, it was shared by geographically isolated necrotic-dysgenesis accessions (i.e., those from the Transcaucasus and Afghanistan–Pakistan border areas) (Fig. 1b). Twenty of the 41 normal accessions had the haplotypes 3, 4, 8, and 11 (Table S2). A Fisher's Exact test showed a significant difference in frequency of these haplotypes between hybrid-abnormality-causing and normal accessions ($P = 4.311\text{e-}06$), indicative that the occurrence of hybrid-abnormality-causing accessions was phylogenetically biased. As expected from their wide geographic distributions, the hybrid-fertilizing accessions had all haplotypes other than number 8, whose solo member was lethal (Fig. 4c). Mean seedset rates and pollen fertility varied between haplotypes but except for the 2005 means for pollen fertility, no significant difference was found (Table 2). Because of the relatively low reproducibility of pollen fertility data (see above), the significant differences found for the 2005 means of pollen fertility require further testing (Table 2, Tukey-Kramer test, $P < 0.05$).

Discussion

Diverse postzygotic reproductive phenotypes

The geographic and phylogenetic analyses of distributions of the *Ae. tauschii* accessions involved in the expression of

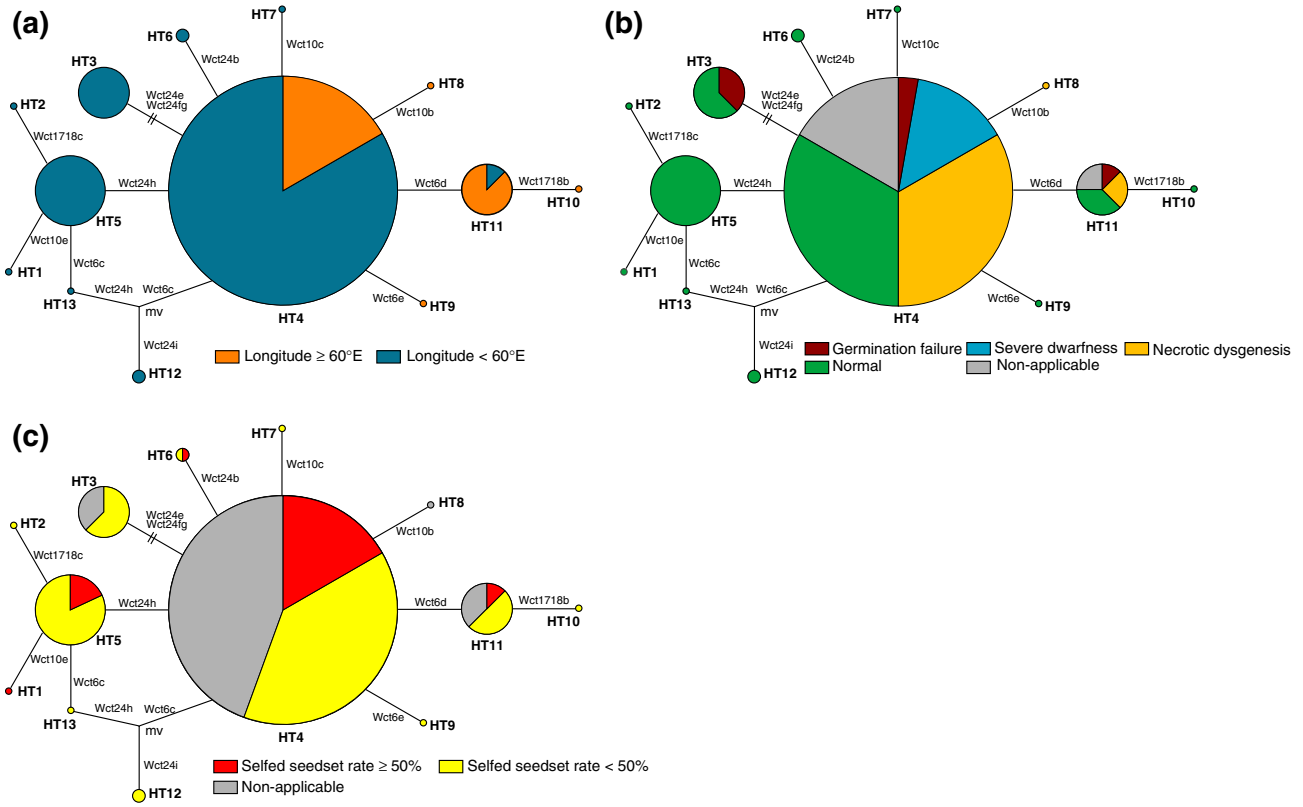

Fig. 4 Reduced-median networks for chloroplast DNA haplotypes. *Circles* denote haplotypes with diameters proportional to the number of accessions. Unless otherwise indicated, haplotypes are connected by branches representing one mutation step. *Two slashes* on an HT3–HT4 branch indicate two mutations. Mutations that define haplotypes are shown on the branches. *mv* denotes a hypothetical haplotype not found in this study. Complex base changes found in WCt24b (Table S5) were treated as one mutation. **a** Colors showing geographic origin proportions. **b** Colors showing hybrid abnormality phenotype proportions. **c** Colors showing hybrid-fertilizing accession proportions

Table 2 Mean seedset rates and pollen fertility of Ldn-*Ae. tauschii* F_1 hybrids

Year and Chloroplast DNA haplotype	No. of accessions	Means, selfed-seed set rates	Standard error	Means, pollen fertility	Standard error
2004					
HP3	3	0.22	0.02	0.43	0.05
HP4	13	0.32	0.04	0.62	0.05
HP5	8	0.33	0.05	0.46	0.07
HP11	2	0.46	0.07	0.84	0.01
2005					
HP3	3	0.27	0.09	0.59^{ab}	0.11
HP4	10	0.46	0.06	0.64^{ab}	0.06
HP5	8	0.49	0.04	0.39^{a}	0.07
HP6	2	0.52	0.09	0.70^{ab}	0.11
HP11	4	0.39	0.09	0.81^{b}	0.03
HP12	2	0.23	0.03	0.84^{b}	0.05

Means were calculated for cpDNA haplotypes that had more than one accession. For the 2005 pollen fertility means, the identical superscript character assigned to mean values indicates no significant difference (Tukey-Kramer test, $P < 0.05$)

postzygotic reproductive phenotypes in the triploid F_1 hybrids with *T. turgidum* showed that (1) hybrid-abnormality-causing accessions have limited geographic and phylogenetic distributions; and (2) hybrid-fertilizing accessions, in contrast, have wide geographic and phylogenetic distributions. These results indicate that postzygotic hybridization barriers are underdeveloped between these species. The diploid *Triticum* and *Aegilops* species radiated 2.5–4.5 MYA (Huang et al. 2002). A pair of the progenitors of current diploid *Triticum* and *Aegilops* species gave rise

to what is now *T. turgidum* 0.5 MYA, whereas, since radiation, *Ae. tauschii* had been isolated from *T. turgidum* until they came into contact about 8,000 years ago (Van Zeist 1976). Contacts occurred as a result of domestication of *T. turgidum* in the Fertile Crescent and the subsequent spread of its cultivated forms, eventually bringing about the formation of hexaploid *T. aestivum* (Zohary and Hopf 2000). This means that *Ae. tauschii* had 2.5 million years or more to develop postzygotic hybridization barriers that prevent formation of fertile hybrids with *T. turgidum*. Considering a pair of animal species that diverged over a comparable amount of time, one would expect complete hybrid inviability, sterility, or both as in *Drosophila* (0.2–2.7 million years) (Coyne and Orr 1997) and mammals (2–3 million years) (Prager and Wilson 1975). The underdeveloped postzygotic hybridization barriers and widespread fertile triploid F_1 hybrids therefore represent a distinct aspect of the evolution of *Triticum* and *Aegilops* species through which diverse expression patterns of postzygotic reproductive phenotypes developed.

Chloroplast DNA phylogenetic analyses made it possible to infer the diversification process of *Ae. tauschii*'s ability to express hybrid-abnormality phenotypes. In this study, cpDNA biallelic base-change and minisatellite sites were used as markers for tracing intraspecific genealogical divergence. Under conditions in which selfing restricts gene flow and recombination, descendents inherit both the nuclear genotype and cpDNA haplotype from the parent and express parental phenotypes if no mutation occurs. This scenario seems to explain the origin of severe-dwarfness accessions that share a single haplotype4 (Fig. 4b). Uniformity of the cpDNA haplotypes together with the narrow geographic distribution (Fig. 1b) suggests a relatively recent, single origin for severe-dwarfness accessions from a haplotype4 parent in the Transcaucasus. In contrast, the coexistence of hybrid-abnormality-causing accessions with normal accessions in a haplotype (Fig. 4b) indicates, assuming reverse mutations were rare, that germination-failure and necrotic-dysgenesis accessions may have arisen recurrently from parental plants that had different cpDNA haplotypes through independent mutations. For example, germination-failure accessions seem to have arisen at least three times; once from a haplotype3 parent (in the western habitats), once from a haplotype11 parent (probably in the eastern habitats), and once from a haplotype4 parent (somewhere). Similarly, necrotic-dysgenesis accessions appear to have arisen at least twice; once from a haploype11 parent (probably in the eastern habitats) and once from a haplotype4 parent (somewhere). The necrotic-dysgenesis-causing ability of the rare haplotype8 seems to have been transmitted from haplotype4 through divergence.

Postzygotic reproductive isolation usually results from complementary genes (Orr 1995), and complementary genes that cause hybrid necrosis and chlorosis have been reported in wheat species (Tsunewaki 1970). Recent *Drosophila* studies showed that allelic mutations in complementary genes that have normal functions within the species cause postzygotic reproductive isolation (Presgraves et al. 2003). In the case of hybrid-abnormality-causing *Ae. tauschii* accessions, their close cpDNA phylogenetic relationships suggest that at many nuclear gene loci they share the same or similar alleles that arose during lineage expansion and diversification through selfing propagation of the ancestral haplotype4 accessions. The phylogenetically biased occurrence of hybrid-abnormality-causing accessions therefore may be the result of sharing haplotype4-lineage-derived alleles that could bring about abnormal phenotypes with a small number of mutations. Such mutations may include those that make *Ae. tauschii*'s complementary gene alleles incompatible with Ldn alleles, as posited by the Dobzhansky-Muller model (Dobzhansky 1936; Muller 1939). In contrast, the absence of hybrid-abnormality-causing accessions in haplotype5 and neighboring ones (haplotypes 1, 2, and 13) suggests that at many nuclear gene loci haplotype5 accessions may have inherited from the ancestral haplotype4 lineage alleles that would need several mutations to become capable of expressing abnormal phenotypes (Fig. 4b). To validate these inferences about the origin and evolution of hybrid-abnormality-causing accessions requires detailed analyses of the genes involved in abnormality expression in hybrids with Ldn.

The wide geographic and phylogenetic distributions of hybrid-fertilizing accessions indicate that this ability evolved through a distinct process. In sharp contrast to hybrid-abnormality-causing accessions, accessions that fertilize the triploid F_1 hybrids with *T. turgidum* seem to have existed during the early stages of the evolution of *Ae. tauschii* and to have spread by dispersal over the species range. To date, the ability to fertilize the hybrids with *T. turgidum* has been well maintained in *Ae. tauschii* populations. With few exceptions, the strengths of this ability do not differ significantly between cpDNA phylogenetic groups, whereas variation does exist among the *Ae. tauschii* genotypes as seen in the degrees of selfed-seedset rate and pollen fertility of F_1 hybrids with *T. turgidum* (Table S3). These findings suggest that the genetic factors involved in hybrid fertility also have a function(s) in *Ae. tauschii* and that mutations that lead to the complete loss of that ability are not beneficial. The expression of hybrid fertility may involve genes that have been conserved for their function(s) during *Ae. tauschii* evolution.

Fukuda and Sakamoto (1992) crossed two of our *Ae. tauschii* accessions (KU-20-9 and KU-20-10) with various accessions of the *T. turgidum* wheats. The seedset rates of their triploid F_1 hybrids varied depending on the *T. turgidum*

genotypes (0.1-23.5% for the KU-20-9 hybrids, 0.4–15.9% for the KU-20-10 hybrids), indicative that *T. turgidum* has genetic factors that affect the fertility of the triploid F_1 hybrids with *Ae. tauschii*. Therefore, the fertility of the Ldn-*Ae. tauschii* triploid F_1 hybrids is probably the product of the mutual action between parental factors. The Ldn-*Ae. tauschii* triploid F_1 hybrids (average selfed-seedset rates, 0.33–0.43 seeds per floret) are more fertile than synthetic haploid Ldn plants (average seedset rate, 2.75 seeds per plant) (Jauhar et al. 2000). This fact appears to indicate that the *Ae. tauschii* factors may increase the Ldn's ability to cause haploid fertility in the triploid genome background.

Ldn has a gene for meiotic restitution (Xu and Joppa 1995, 2000). A triploid F_1 hybrid between Ldn and an accession of *Ae. tauschii* produces 2*n* gametes through unreduced meiosis (Matsuoka and Nasuda 2002). The observed hybrid fertility variation might be associated with the ability to meiotically produce 2*n* gametes. Details of the underlying mechanism for fertility of triploid *T. turgidum–Ae. tauschii* F_1 hybrids, however, have yet to be addressed.

Impact on allopolyploid speciation

Our findings shed light on the impact of natural variation in the ability of parental species to express postzygotic reproductive phenotypes on allopolyploid speciation. In *T. aestivum* speciation, our systematic-cross experiment showed narrow geographic and phylogenetic distributions for hybrid-abnormality-causing accessions but did not provide evidence that hybrid abnormality had a major impact. In contrast, geographic and phylogenetic distribution patterns of hybrid-fertilizing accessions suggest that they may have driven speciation of *T. aestivum* widely in nature and negate the view that only particular populations of *Ae. tauschii* had such ability and consequently participated in speciation. Many naturally occurring *Ae. tauschii* populations most likely retain the ability to give rise to fertile triploid F_1 hybrids with *T. turgidum* cultivars that genetically are similar to Ldn. We speculate that should F_1 hybrids be formed between a Ldn-like *T. turgidum* and *Ae. tauschii* under natural conditions, the possibility that such hybrids would grow normally and set hexaploid F_2 seeds is reasonably high. The wide geographic distribution of the hybrid-fertilizing accessions thus provides empirical support for the theory that allopolyploid *T. aestivum* speciation took place at multiple sites within the species range of *Ae. tauschii*, in which conditions enabled natural hybridization with cultivated *T. turgidum* (Salamini et al. 2002). More than one origin of *T. aestivum* wheats has been suggested based on their phenotypic and genetic diversities (Mackey 1966; Talbert et al. 1998; Caldwell et al. 2004, Giles and Brown 2006), whereas introgression from emmer wheat is likely to have had a role in the diversification of *T. aestivum* wheats (Hirosawa et al. 2004).

To better understand the origin and evolution of *T. aestivum* wheats, the impact of hybrid abnormality needs to be assessed with other genotypes of *T. turgidum* as testers. Genetic analyses are required to test whether the expression of the severe dwarfness and necrotic dysgenesis phenotypes of Ldn-*Ae. tauschii* F_1 hybrids involves the known complementary gene systems (Nishikawa 1960, 1962; Hermsen 1967; Tsunewaki 1970). Furthermore, our findings invite studies of the roles of prezygotic factors in the allopolyploid speciation of *T. aestivum.* Prezygotic factors have high potential for affecting the tempo, mode, and geography of allopolyploid *T. aestivum* speciation by preventing or promoting natural hybridization crosses that would lead to the birth of fertile triploid F_1 hybrids capable of setting hexaploid F_2 seeds. In fact, prezygotic barriers, are the major reproductive isolation components in plants and animals (Coyne and Orr 1997; Ramsey et al. 2003). To evaluate the impact of prezygotic factors on the allopolyploid *T. aestivum* speciation requires agro ecological studies that integrate biological, anthropological, and environmental approaches because the cradles of *T. aestivum* evolution were the wheat fields brought under cultivation by early Middle Eastern agriculturists.

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References

Bandelt HJ, Forster P, Sykes BC, Richards MB (1995) Mitochondrial portraits of human populations using median networks. Genetics 141:743–753

Caldwell KS, Dvorak J, Lagudah ES, Akhunov E, Luo MC, Wolters P, Powell W (2001) Sequence polymorphism in polyploid wheat and their D-genome diploid ancestor. Genetics 167:941–947

Coyne JA, Orr HA (1997) "Patterns of speciation in *Drosophila*" revisited. Evolution 51:295–303

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Massachusetts, pp 9–54

Dobzhansky T (1936) Studies on hybrid sterility. II. Location of sterility factors in *Drosophila pseudoobscura* hybrids. Genetics 21:113–135

Doebley J, Stec A (1991) Genetic analysis of the morphological differences between maize and teosinte. Genetics 129:285–295

Dvorak J, Luo MC, Yang ZL, Zhang HB (1998) The structure of the *Aegilops tauschii* genepool and the evolution of hexaploid wheat. Theor Appl Genet 97:657–670

Fukuda K, Sakamoto S (1992) Studies on the factors controlling the formation of unreduced gametes in hybrids between tetraploid emmer wheats and *Aegilops squarrosa* L. Japan J Breed 42:747–760

Giles RJ, Brown TA (2006) *GluDy* allele variations in *Aegilops tauschii* and *Triticum aestivum*: implications for the origins of hexaploid wheats. Theor Appl Genet 112:1563–1572

Hermsen JGT (1967) Hybrid dwarfness in wheat. Euphytica 16:134–162

Hirosawa S, Takumi S, Ishii T, Kawahara T, Nakamura C, Mori N (2004) Chloroplast and nuclear DNA variation in common wheat: insight into the origin and evolution of common wheat. Genes Genet Syst 79:271–282

Huang S, Sirikhachornkit A, Su X, Faris J, Gill B, Haselkorn R, Gornicki P (2002) Genes encoding plastid acetyl-CoA carboxylase and 3-phosphoglycerate kinase of the *Triticum/Aegilops* complex and the evolutionary history of polyploid wheat. Proc Natl Acad Sci USA 99:8133–8138

Ishii T, Mori N, Ogihara Y (2001) Evaluation of allelic diversity at chloroplast microsatellite loci among common wheat and its ancestral species. Theor Appl Genet 103:896–904

Jauhar PP, Dogramaci-Altuntepe M, Peterson TS, Almouslem AB (2000) Seedset on synthetic haploids of durum wheat: cytological and molecular investigations. Crop Sci 40:1742–1749

Kihara H (1944) Discovery of the DD-analyser, one of the ancestors of *Triticum vulgare* (abstr) (in Japanese). Agric Hortic 19:889–890

Kihara H, Lilienfeld F (1949) A new synthesized 6x-wheat. Hereditas (Suppl): 307–319

Kihara H, Yamashita K, Takaka M (1965) Morphological, physiological, genetical and cytological studies in *Aegilops* and *Triticum* collected from Pakistan, Afghanistan and Iran. In: Yamashita K (ed) Results of the Kyoto University Scientific Expedition to the Karakoram and Hindukush, 1955, vol. I. Kyoto University, Kyoto, pp 1–118

Mackey J (1966) Species relationship in *Triticum*. Hereditas (Suppl 2):237–276

Masterson J (1994) Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. Science 264:421–424

Matsuoka Y, Nasuda S (2004) Durum wheat as a candidate for the unknown female progenitor of bread wheat: an empirical study with a highly fertile F_1 hybrid with *Aegilops tauschii* Coss. Theor Appl Genet 109:1710–1717

Matsuoka Y, Mori N, Kawahara T (2005) Genealogical use of chloroplast DNA variation for intraspecific studies of *Aegilops tauschii* Coss. Theor Appl Genet 111:265–271

McFadden ES, Sears ER (1944) The artificial synthesis of *Triticum spelta*. Rec Genet Soc Am 13:26–27

Muller HJ (1939) Reversibility in evolution considered from the standpoint of genetics. Biol Rev Camb Philos Soc 14:261–280

Nishikawa K (1960) Hybrid lethality in crosses between emmer wheats and *Aegilops squarrosa*, I. Vitality of F_1 hybrids between emmer wheats and *Ae. squarrosa* var. *typica*. Seiken Ziho 11:21–28

Nishikawa K (1962) Hybrid lethality in crosses between emmer wheats and *Ae. squarrosa*, II. Synthesized 6x wheats employed as test varieties. Jpn J Genet 37:227–236

Orr HA (1995) The population genetics of speciation: the evolution of hybrid incompatibilities. Genetics 139:1805–1813

Prager EM, Wilson AC (1975) Slow evolutionary loss of the potential for interspecific hybridization in birds: a manifestation of slow regulatory evolution. Proc Natl Acad Sci USA 72: 200–204

Presgraves DC, Balagopalan L, Abmayr SM, Orr HA (2003) Adaptive evolution drives divergence of a hybrid inviability gene between two species of *Drosophila*. Nature 423:715–719

Ramsey J, Schemske DW (2002) Neopolyploidy in flowering plants. Annu Rev Ecol Syst 33:589–639

Ramsey J, Bradshaw HD, Schemske DW (2003) Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57:1520–1534

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Salamini F, Özkan H, Brandolini A, Schäfer-Pregl R, Martin W (2002) Genetics and geography of wild cereal domestication in the Near East. Nature Rev Genet 3:429–441

Soltis DE, Soltis PS, Tate JA (2003) Advances in the study of polyploidy since *Plant Speciation*. New Phytol 161:173–191

Talbert LE, Smith LY, Blake NK (1998) More than one origin of hexaploid wheat is indicated by sequence comparison of low-copy DNA. Genome 41:402–407

Tanaka M, Tsujimoto H (1991) Natural habitat of *Aegilops squarrosa* in Xinjiang Uygur, China. Wheat Inf Serv 73:33–35

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

Tsunewaki K (1970) Necrosis and chlorosis genes in common wheat and its ancestral species. Seiken Ziho 22:67–75

Van Slageren MW (1994) Wild wheats: a monograph of *Aegilops* L. and *Amblyopyrum* (Jaub. & Spach) Eig (Poaceae). Wageningen Agricultural University, Wageningen, pp 326–344

Van Zeist W (1976) On macroscopic traces of food plants in southwestern Asia (with some reference to pollen data). Phil Trans R Soc Lond B 275:27–41

Xu SJ, Joppa LR (1995) Mechanisms and inheritance of first division restitution in hybrids of wheat, rye, and *Aegilops squarrosa*. Genome 38:607–615

Xu SJ, Joppa LR (2000) First-division restitution in hybrids of Langdon durum disomic substitution lines with rye and *Aegilops squarrosa*. Plant Breed 119:233–241

Zohary D, Hopf M (2000) Domestication of plants in the Old World, 3rd edn. Oxford University Press, Inc., New York, pp 51–58