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Research

Long-term persistence of crop alleles in weedy populations of wild radish (*Raphanus raphanistrum*)

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Summary

- Hybridization allows transgenes and other crop alleles to spread to wild/weedy populations of related taxa. Researchers have debated whether such alleles will persist because low hybrid fitness and linkage to domestication traits could severely impede introgression.
- To examine variation in the fates of three unlinked crop alleles, we monitored four experimental, self-seeding, hybrid populations of *Raphanus raphanistrum* × *Raphanus sativus* (radish) in Michigan, USA, over a decade. We also compared the fecundity of advanced-generation hybrid plants with wild plants in a common garden experiment.
- Initially, F_1 hybrids had reduced fitness, but the populations quickly evolved wild-type pollen fertility. In Year 10, the fecundity of plants from the experimental populations was similar to that of wild genotypes. Crop-specific alleles at the three loci persisted for 10 yr in all populations, and their frequencies varied among loci, populations and years.
- This research provides a unique case study of substantial variation in the rates and patterns of crop allele introgression after a single hybridization event. Our findings demonstrate that certain crop alleles can introgress easily while others remain rare, supporting the assumption that neutral or beneficial transgenes that are not linked to maladaptive traits can persist in the wild.

Introduction

A common concern about transgenic crops is that novel, fitness-enhancing transgenes may spread to wild/weedy relatives, perhaps resulting in greater weed problems or harm to nontarget organisms (Snow *et al.*, 2005; Andow & Zwahlen, 2006). Transgenes are expected to introgress into wild populations in the same manner as conventional crop alleles (Ellstrand, 2003). Some crop alleles might be purged from wild populations via outbreeding depression or selection against maladaptive crop traits, such as lack of seed dormancy (Gressel, 1999; Warwick & Stewart, 2005). However, other alleles that are neutral or beneficial may persist if they are not tightly linked to detrimental alleles (Ellstrand, 2003). Thus, we expect the evolutionary trajectory of introgressing crop alleles to vary owing to outbreeding depression, fitness effect, linkage, and any heterosis that results from greater heterozygosity in hybrid lineages.

Researchers have long known that cultivated plants can hybridize spontaneously with their wild/weedy relatives (de Wet & Harlan, 1975; Ellstrand, 2003; Martinez-Castillo *et al.*, 2007), but rigorous studies of introgression (i.e. the stable incorporation of crop genes into the wild gene pool) and its ecological consequences are scant. Here, we use the terms 'wild' and 'weedy' interchangeably because wild relatives that occur near agricultural crops may represent a crop–wild–weed complex of interbreeding taxa (de Wet & Harlan, 1975; Ellstrand, 2003). Several previous studies showed that crop alleles appear to persist in populations of wild relatives. In sunflower, Whitton *et al.* (1997) documented the persistence of two RAPD markers from cultivated sunflower in a single nearby population of wild sunflowers over 5 yr. Also in sunflower, Linder *et al.* (1998) identified putatively crop-specific random amplified polymorphic DNA (RAPD) markers that were rare or absent in four allopatric wild populations, but were common in three

wild populations near cultivated fields. Morrell *et al.* (2005) found putatively crop-specific alleles from cultivated sorghum (*Sorghum bicolor*) in adjacent and disjunct populations of johnsongrass (*Sorghum halepense*), a widespread noxious weed. In each of these examples, there is a small chance that the putatively crop-specific alleles were already present in recipient populations because of the shared ancestry of wild plants, cultivars and their weedy relatives (see Chapter 5 of Ellstrand, 2003).

Transgenes can provide unambiguous molecular markers for assessing crop-to-wild gene flow, although only at the locus of insertion. For example, transgenes that confer resistance to glyphosate and glufosinate have been found in volunteer and feral populations of canola (*Brassica napus*) owing to both pollen- and seed-mediated gene flow (Hall *et al.*, 2000; Aono *et al.*, 2006). In Sweden, transgenic seeds of *B. napus* germinated 10 yr after a field trial conducted in 1995 (D'Hertefeldt *et al.*, 2008). Hybridization can occur between *B. napus* and weedy *Brassica rapa*, despite reduced fitness and differences in ploidy level (Jørgensen & Andersen, 1994; Hauser *et al.*, 2003). Four years after transgenic F_1 hybrids between cultivated *B. napus* and wild *B. rapa* were found at two sites in Canada (Warwick *et al.*, 2003), transgenic resistance to glyphosate was detected in a single individual of *B. rapa* (Warwick *et al.*, 2008), suggesting that longer term persistence could occur in this region. In another well-studied example, the accidental escape of pollen and seeds from transgenic creeping bentgrass (*Agrostis stolonifera*) led to inadvertent gene flow to wild populations in Oregon, USA, before regulatory approval (Watrud *et al.*, 2004; Reichman *et al.*, 2006; Zapiola *et al.*, 2008). Transgenic wild plants were found 3 yr after pre-commercial fields of transgenic bentgrass were discontinued (Zapiola *et al.*, 2008). The results of these studies are consistent with the expectation that selectively neutral or beneficial transgenes may persist in crop volunteers and wild/weedy populations.

The purpose of the current study was to examine changes in the frequencies of three unambiguous, crop-specific alleles in crop–weed hybrid populations over many generations. Using a novel experimental approach that employed naturally occurring, crop-specific markers, we established four replicated wild populations in which the frequencies of crop alleles were monitored for 10 yr following a single episode of hybridization. We found that alleles from cultivated radish (*Raphanus sativus*) persisted, but with different evolutionary trajectories, in populations of wild radish (*Raphanus raphanistrum*, also known as 'jointed charlock'). We also document an initial reduction in fitness in hybrids relative to wild plants and subsequent recovery of wild-type pollen fertility and fecundity in advanced-generations. In a separate experiment, we show that fitness components of F_2 crop–wild hybrids did not vary as a result of the sources of wild or cultivar seeds, which supports the generality of our long-term study.

Materials and Methods

Study system

Raphanus raphanistrum L. is a self-incompatible, annual weed of Eurasian origin (Holm *et al.*, 1997). This species occurs in agricultural fields, waste places, and along sheltered beaches in many cool/temperate areas of the world. *Raphanus raphanistrum* seeds are dispersed as contaminants of grain, and the weed is especially troublesome in wheat, oat, alfalfa, and barley fields (Cheam & Code, 1995; Weaver & Ivany, 1998; Fischer *et al.*, 1999; Cousens *et al.*, 2001). *Raphanus raphanistrum* often hybridizes with cultivated radishes (*R. sativus* L.), which are also self-incompatible, in areas where these taxa co-occur (Stace, 1975; Snow & Campbell, 2005). Both types of *Raphanus* are pollinated by a variety of insects, including bumblebees, halictid bees, syrphid flies, honeybees, and butterflies (Stanton, 1987; Lee & Snow, 1998; Sahli & Conner, 2007).

Interspecific hybrids between *R. raphanistrum* and cultivated *R. sativus* are vigorous and fertile (both species have $2n = 2x = 18$ chromosomes), although F_1 hybrids typically have *c.* 50–60% aborted pollen grains (Panetsos & Baker, 1967) and produce fewer seeds per fruit than wild genotypes (Snow *et al.*, 2001). Low pollen fertility in hybrids has been attributed to heterozygosity for a reciprocal translocation that affects chromosome pairing during meiosis (Panetsos & Baker, 1967). Thus, low pollen fertility can serve as indirect evidence for recent hybridization between the crop and its wild relative, at least until one version of the translocation becomes fixed in a given population. In California, where the crop and the weed were introduced in the late 1800s, free-living *Raphanus* populations are widespread and most, if not all, appear to be hybrid-derived; apparently, pure *R. raphanistrum* is no longer present (Panetsos & Baker, 1967; Hegde *et al.*, 2006).

In Michigan, where the current study was conducted, *R. raphanistrum* occurs as a weed in alfalfa, oats, potato, sugar beet and other agricultural fields, and it is common in the seed bank of former agricultural land (A. A. Snow and L. G. Campbell, pers. obs.). Some populations of adult plants persist for many consecutive years within cultivated areas and adjacent disturbed areas, while others recruit sporadically from extensive seed banks. In Michigan and elsewhere, cultivated radish is grown in home gardens and commercial fields, with the potential to hybridize with the weed when the crop is grown for its seeds or when fields are neglected and the rosettes bolt to produce flowers.

The crop is homozygous for the dominant, white petal allele, whereas most *R. raphanistrum* plants are homozygous for the recessive, yellow carotenoid pigment (Panetsos &

Baker, 1967; Kay, 1976; Stanton *et al.*, 1986). Cultivated radish is also polymorphic for anthocyanin-based, pink petal coloration at another locus (Irwin *et al.*, 2003). In this research, we studied only carotenoid variation; pink-flowered plants were grouped with white-flowered ones, and bronze-flowered plants (i.e. pink blended with yellow) were grouped with yellow-flowered plants to monitor allele frequencies at the white/yellow locus (as in Snow *et al.*, 2001). Indirect evidence for crop-to-wild gene flow in Michigan comes from the presence of crop-specific flower colors (white, pink and bronze), as reported for a Bay City, MI, USA, population of *R. raphanistrum* by Kercher & Conner (1996). In this population, 55% of the plants had crop-specific flower colors, and we have often observed lower frequencies of putative hybrids in other populations (A. A. Snow, pers. obs.).

Crop allele persistence in experimental populations

Genetic markers and seed sources We did not intend to monitor genome-wide, crop-specific markers in this study. Rather, we chose three genetic markers – flower color and two allozymes – that could be tracked relatively inexpensively and easily. Wild radish plants were selected for crosses based on specific alleles of GPI (glucose-6-phosphate isomerase; EC 5.3.1.9; four alleles) and PGM (phosphoglucomutase; EC 5.4.2.2; three alleles) that were distinct from alleles found in the common cultivar Scarlet Globe. These loci are not linked and therefore represent independent genetic markers in *R. raphanistrum* (Conner *et al.*, 1997) and in *R. sativus* (Ellstrand & Devlin, 1989). In addition they do not appear to be linked to the white/yellow petal locus based on different patterns of persistence (see Results). We did not make any *a priori* assumptions about whether these three markers had an impact on fitness or were selectively neutral (see the Discussion section).

Wild seeds from our Michigan collections could not be used for establishing the baseline populations because the GPI and PGM alleles that were most common in the crop (GPI-3 and PGM-1 or PGM-3) were not sufficiently rare in Michigan wild plants. Instead we used wild plants from a population in Ocean Point, ME, USA (same population as in Kercher & Conner, 1996). For simplicity, we combined data from PGM-1 and PGM-3 in reporting our results on crop-specific alleles. We assumed that these non-local wild plants would not differ greatly from Michigan ones as wild or hybrid phenotypes. Wild populations from Maine and Michigan did not differ in fecundity or pollen fertility when compared in a common garden in Michigan (see the Results section). Also, levels of genetic differentiation among populations of *R. raphanistrum* in Maine, Michigan and other areas in the USA are moderate and are not related to geographic distance, most likely because the seeds are dispersed widely as contaminants of grain (Kercher & Conner, 1996; Sahli *et al.*, 2008).

Seeds for the experimental populations were obtained by pollinating 17 wild plants with pollen from each of eight 'Scarlet Globe' crop plants or with pollen from each other in a glasshouse. Progeny from these parental plants were checked for expected allozyme alleles and were equally distributed among the four experimental populations.

Establishing the populations Detailed methods for establishing the populations are described in Snow *et al.* (2001) and are summarized here. Four isolated, self-seeding populations were established at the University of Michigan Biological Station (UMBS), in Pellston, MI, USA, in 1996. The populations were designated as 1–4, which are the same as Populations BS, RR, GM and PP, respectively, in Snow *et al.* (2001). The populations were separated by at least 2 km and were > 5 km from the nearest populations of *R. raphanistrum* and cultivated radishes, neither of which are common in Pellston. At each field site, a 15 × 15 m area was cleared, rototilled, and fenced to exclude deer.

F_1 crop–wild hybrids were intermixed with wild plants to simulate the initial outcome of spontaneous hybridization, using 100 wild plants and 100 F_1 crop–wild hybrids per population. Under natural conditions, frequencies of crop–wild hybrids are likely to vary widely, depending on the relative sizes of source and sink populations and the predominant directions of pollen flow (i.e. crop-to-wild or wild-to-crop) (see Klinger *et al.*, 1991, 1992). We chose an initial frequency of 0.25 to ensure that crop alleles would not be too rare to be detected with future sample sizes of *c.* 150–200 plants per population.

Two-week-old seedlings were planted during the first week of July, and we hand-weeded a 10 cm radius around each plant during the first 3 wk of Year 1 to encourage successful establishment. Each plant also received 3.5 g of slow-release Osmocote fertilizer on July 6th and July 22nd. The populations were exposed to local pollinators, herbivores, pathogens, competing vegetation, and environmental conditions. At the end of the growing season, the fruits fell to the ground and overwintered *in situ*. To mimic agricultural conditions, we rototilled each site in the spring and, starting in 1999, we added equal amounts of fertilizer to each site.

Data collection Each year, we estimated the size of each population at the time of peak flowering by counting all plants within 35–50 evenly spaced quadrats (Snow *et al.*, 2001). If fewer than 400 plants were present, we counted every plant. We calculated the average number of plants per quadrat and multiplied by the total area to estimate population size. We acknowledge that *R. raphanistrum* has a persistent seed bank (Warwick & Francis, 2005), but we did not attempt to estimate it for this study.

Flower color frequencies were counted within the quadrats, or by counting every plant when there were fewer than 400 individuals (see Table 1 for sample sizes). Samples for allozyme analyses were collected from leaves (Generations 1, 3, and 4) or seeds from *c.* 200 haphazardly chosen plants per population. We obtained allele frequencies for PGM and GPI using starch gel electrophoresis on histidine-citrate gels (pH 6.0) run for 4–5 h or overnight at 150–170 V, following methods in Kercher & Conner (1996).

Pollen fertility was assessed as in Snow *et al.* (2001). During the first year, we collected pollen samples from a total of 20 wild plants and 20 hybrids from two of the four populations. We found that 95% of the wild plants had at least 70% fertile pollen, and this threshold was used for comparing pollen fertility among years and populations (sample sizes in Table 1).

Statistical analyses and simulations To test for significant differences in frequencies of crop-specific alleles and pollen fertility among populations and years, we analysed data from each –year combination in two-way ANOVAs. Because flower color is a dominant trait, we omitted data from Year 1. Similarly, for pollen fertility, we omitted data from Year 1 because all F_1 hybrids were heterozygous for the reciprocal translocation that causes low fertility. Similar results were obtained when data from Year 1 were included in these analyses (not reported).

Using PROC MIXED (SAS 2003), population was considered as a fixed factor, and year was either fixed or random. Both methods gave similar results and we report results from considering year as fixed. To test whether crop-specific allozyme frequencies or the proportion of white-flowered plants declined over time, we ran a Type III repeated-measures ANOVA in which population was a fixed factor and year was the repeated measure (defining population as a random effect did not change our conclusions).

To test for deviations from genetic drift, we used simulations and compared observed frequencies of crop-specific alleles with expected values based on known fluctuations in the sizes of the four populations over time. The simulations were based on a single locus with two allele types, crop or wild, using C programming language. We assumed that individuals with different genotypes at this locus had equal fitness and mated randomly. The simulation generated

Table 1 Population sizes (number of flowering plants) and sample sizes for genetic markers and pollen fertility in the four experimental populations

Generation	1	2	3	4	5	6	7	8	9	10
Year	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Population size										
Population 1	184	2805	14 380	101 000	7440	2071	7459	8845	42 336	2552
Population 2	188	2127	3770	86 000	20 555	2184	21 243	6624	12 766	11 746
Population 3	177	303	164	3592	21 315	4808	4739	8585	11 731	15 554
Population 4	174	237	58	1123	65 425	6058	30 633	12 848	27 069	24 318
GPI alleles										
Population 1	184	–	218	142	–	217	179	236	–	162
Population 2	188	–	140	230	–	212	210	148	–	179
Population 3	177	–	–	138	–	210	206	162	–	186
Population 4	174	–	–	153	–	168	208	207	–	173
PGM alleles										
Population 1	184	–	237	151	–	217	179	226	–	210
Population 2	188	–	152	238	–	212	210	278	–	187
Population 3	177	–	–	139	–	210	206	189	–	207
Population 4	174	–	–	153	–	168	208	213	–	210
Flower color										
Population 1	184	273	3177	955	1439	209	919	1191	1683	703
Population 2	188	410	1009	1004	1205	455	2607	1129	1269	911
Population 3	177	116	164	457	1591	880	731	1073	1340	1695
Population 4	174	126	58	361	4261	1039	3655	1399	1579	1882
Pollen fertility										
Population 1	–[2]	34	60	100	–	90	–	100	–	42[3]
Population 2	–[2]	47	59	102	–	100	–	94	–	38[3]
Population 3	–[2]	48	60	99	–	81	–	104	–	39[3]
Population 4	–[2]	50	48	100	–	100	–	100	–	39[3]

In 1996, each population consisted of 200 seedlings, half of which were F_1 crop–wild hybrids. Numbers of flowering plants were lower than this, primarily owing to delayed flowering of hybrids.

[1]Dashes in Table indicate no data.

[2]See Fig. 1 and text for details on sample collection in 1996 (20 wild plants, 20 hybrid plants).

[3]Data from common garden plants in Table 4.

diploid genotypes for the next generation by randomly selecting two alleles per individual in proportion to their frequencies in the previous year, step by step, for 10 generations. We ran the simulations 1000 times per population. When predicted allele frequencies were higher or lower than observed frequencies for 95% of the runs, we concluded that the allele frequencies differed significantly from expectations for genetic drift. The initial frequency of the crop allele was 0.25, and population sizes varied as shown in Table 1 over 10 yr. Average expected frequencies ranged from 0.248 to 0.253 based on 1000 simulations and known population sizes, assuming random mating and no fitness differences associated with crop alleles; by Year 10, standard deviations from average expected frequencies ranged from 0.025 to 0.052. For the flower color locus, we estimated allele frequencies based on Hardy–Weinberg assumptions, with the caveat that actual allele frequencies at this locus could differ whenever conditions for Hardy–Weinberg equilibrium are not met.

Recovery of fecundity and pollen fertility in hybrid populations

To compare advanced-generation plants with wild genotypes, we grew potted plants in a common garden at UMBS. Tenth-generation seeds were randomly collected from 40–50 plants per population (one seed per plant; see Table 4 for sample sizes). Wild seeds came from two sources – a local population in Pellston, MI, USA, and a wild population from Deer Isle, ME, USA (not enough seeds from the parent population in Ocean Point, ME, were available for this experiment). Because plants from the wild populations in Michigan and Maine had similar survival, fecundity and pollen fertility (see the Results, Table 4), we assume that differences in their genetic background and maternal environments were not strong enough to be important in this study.

This experiment was carried out using the same procedures as in Campbell *et al.* (2006). We used a randomized block design with 21 blocks and a total of 31–43 plants in each of the six groups (four experimental hybrid populations and two wild populations). Seeds were planted in 300 ml of PRO-MIX 'BX' peat in biodegradable pots in a glasshouse in early May 2005, with two cultivated spring oat seeds (*Avena sativa*) in each pot to provide a uniform level of competition. The garden area was cleared of vegetation and rototilled twice. After the radish seedlings developed their first true leaves, we transplanted each pot into a section of PVC tube (46 cm tall) filled with 1.7 l of local soil. The bottom of tube pot was enclosed with mesh screening that allowed roots to grow into the soil below. The tube pots were separated by 30 cm to reduce root competition among neighbors. Plants were watered daily for the first month and every other day until August 31. On June 18 we added 13 mg of fertilizer (slow-release Osmocote; Scotts Miracle-Gro Co., Marysville, OH, USA) to each pot to supplement the sandy and nutrient poor soil. Insecticide (0.0033% esfenvalerate, 20 g per 9.5 l; Scotts Miracle-Gro Co., Marysville, OH, USA) was used to control insect herbivory three times during the first month, when herbivory was highest. Aphids were present at low densities later in the season but did not colonize any plant heavily. Pollinators were abundant throughout the experiment, as in Lee & Snow (1998).

All plants survived to flower, so survival was not considered further. Pollen fertility was assessed as described earlier. After the plants senesced, we counted numbers of flower pedicels and fruits per plant. To estimate the number of seeds per plant, we multiplied the average number of seeds per fruit (for 10 randomly chosen fruits per plant) by the number of fruits. The proportion of fertile pollen grains per plant was arcsine, square-root transformed; seed data were natural log transformed. We ran a linear mixed model ANOVA for each fitness component using PROC MIXED (SAS, 2003); population was considered as a fixed effect and block was a random effect.

Comparisons of hybrid and wild progeny from different seed sources

To assess the generality of our long-term study, which was based on one wild parent population and one cultivar parent, we compared fitness components of F_2 progeny from four wild populations and four cultivars in a second common garden plot. We obtained seeds from 100 to 200 wild radish plants per population from Leesburg, GA, Binghamton, NY; Pellston, MI, and Deer Isle, ME, USA. Crop pollen was collected from *c.* 50 plants from each of four common radish cultivars: Cherriette, Cabernet, Scarlet Globe (Stokes Seeds, Inc., Fredonia, NY, USA) and Red Silk (Harris-Moran Seed Co., Modesto, CA, USA). In a glasshouse, we hand-pollinated *c.* 50 wild plants with either wild pollen from the same population to create F_1 wild biotypes or crop pollen to create F_1 crop–wild hybrid biotypes, using methods similar to Campbell *et al.* (2006).

These crosses were designed to allow comparisons among hybrid progeny of one wild population (MI) crossed with each of the four cultivars, and among hybrid progeny of one cultivar (Scarlet Globe) crossed with each of the four wild populations and their respective wild parents. A group of *c.* 100 randomly selected F_1 plants per cross type was used to generate the F_2 generation from hand-pollination in the glasshouse. The F_2 plants were grown outdoors in a randomized block design common garden adjacent to the one described earlier, following similar protocols. All plants survived to flower, so survival was not considered further.

Three statistical analyses were used to assess differences in fecundity and pollen fertility among; F_2 progeny from the wild populations, F_2 crop–wild progeny from 'Scarlet

Globe' crossed with different wild parents, and F_2 crop–wild progeny from Michigan crossed with different cultivar parents. For these tests, wild population, cultivar parent, or wild parent were considered to be random variables and we used PROC MIXED (SAS, 2003), followed by LSD comparisons using PROC GLM (with population considered as fixed). We also performed preplanned LSD tests to identify significant differences between each F_2 hybrid and its associated wild parent.

Results

Fluctuations in population size

In 1996, survival of the founding plants was high (97–100%), but fewer F_1 hybrids reproduced than wild plants because of delayed flowering (Snow *et al.*, 2001). This difference was greatest at Populations 3 and 4, where the plants grew more slowly and only 60–62% of the hybrids produced fruits, compared with 92–95% of the wild plants. By contrast, 71–78% of the hybrids produced fruits in Populations 1 and 2, compared with 95–97% of the wild plants (Snow *et al.*, 2001). These early differences in the relative fitness of hybrids may help explain the lower frequencies of white petal alleles in Populations 3 and 4, as discussed later.

A related difference among the four populations is the low recruitment observed in Populations 3 and 4 in 1997 and 1998 (Table 1). In 1996, the founding plants at these sites were smaller than at Populations 1 and 2, apparently owing to the lower organic content of the sandy soil. By the early summer of 1998, seedling densities at Populations 3 and 4 were very low, and grasshoppers killed so many seedlings that it was necessary to transplant and rear the plants in a safer location to prevent extinction. We rescued *c.* 200 seedlings from Population 3 and *c.* 80 seedlings from Population 4 by moving them to pots at another site. Plants from Population 3 were replanted before flowering, whereas those from Population 4 flowered at an isolated location *c.* 0.5 km from their original site and were replanted in September. Thus, by the end of the growing season there were only 158 reproductive plants in Population 3 and 58 in Population 4, plus an unknown number of F_2 seeds that may have remained dormant in the soil during the growing season. Population sizes at all of the sites increased dramatically after we began fertilizing them in 1999 (Table 1), to better mimic agricultural conditions. For the remainder of the study, annual fluctuations in population size appeared to be influenced mainly by competition with other weedy species.

Persistence of crop-specific markers

The four populations began with crop allele frequencies of 0.25 and retained the crop-derived alleles for 10 yr, with considerable variation among loci, years and locations (Fig. 1). The ANOVAs showed that GPI frequencies varied significantly among years, PGM frequencies varied among populations, and the frequency of white-flowered plants varied among both years and populations (Table 2). To determine whether such variation could be attributed to genetic drift, we used simulations to compare observed and expected frequencies, assuming random mating, no differences in immigration or emigration, and no differences in fitness among genotypes. The crop allele for GPI was often significantly less frequent than expected in all four populations (Table 3). By contrast, trajectories for crop-specific PGM alleles differed among populations. The frequency of this marker declined significantly in Population 2 in all years, but remained similar to genetic drift expectations in Populations 3 and 4 (with one exception in Year 6). In Population 1, crop alleles of PGM were significantly lower than expected in Years 3 and 7, and were higher than expected in Year 8 (Table 3).

Frequencies of the dominant, crop-specific allele for white petals showed a strong initial decline and remained much lower than the allozyme markers, but this allele persisted in all populations (Fig. 1). Allele frequencies for the dominant white petal allele can be approximated based on Hardy–Weinberg expectations using plant frequencies. In all years and populations, observed allele frequencies were significantly lower than expected (Table 3), and for the tenth generation, estimated frequencies of the white petal allele averaged only 0.04 across sites. Populations 3 and 4 had very low frequencies of white-flowered plants, following unusually low fitness of F_1 hybrids in Year 1. This difference among populations persisted throughout the 10 yr of our study (Fig. 1). These two populations were maintained for 4 yr beyond the 10-yr study, through 2009, and they retained white-flowered plants in each subsequent year (A. A. Snow, pers. obs.; frequencies not recorded).

Recovery of pollen fertility and wild-type fecundity

The four populations regained wild-type pollen fertility within *c.* 3–6 yr (Fig. 1). The common garden experiment showed that F_{10} plants had similar pollen fertility, flower number and seed production compared with plants from two wild populations (Table 4). In this experiment, none of the differences among populations were statistically significant, with the exception of Population 4, where the average pollen fertility of hybrids was greater than that of the two wild populations ($P < 0.05$, Table 4).

Comparisons among F_2 hybrid progeny from different wild and cultivar parents

No significant differences were seen in pollen fertility, flower number, or fecundity of F_2 progeny in comparisons among F_2 hybrids from different cultivars or among F_2

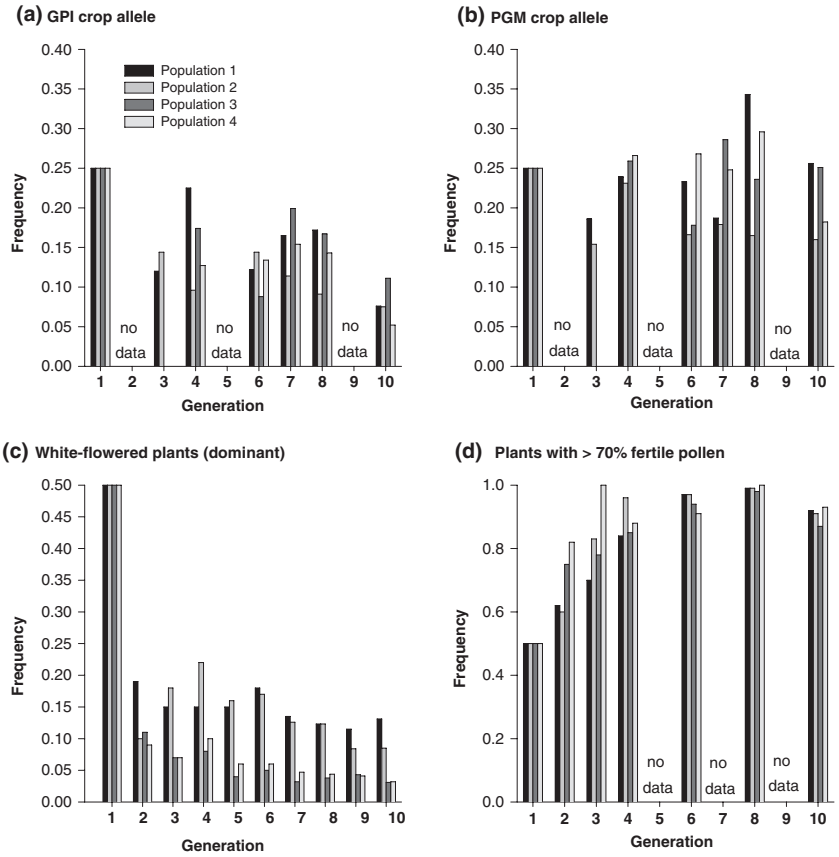

Fig. 1 Frequencies of crop-specific, genetically based markers and recovery of pollen fertility in four experimental wild radish (*Raphanus raphanistrum*) populations. See Table 1 for sample sizes. Data for 1996 (Generation 1 = Year 1) are based on 100 wild and 100 F_1 crop–wild hybrid seedlings planted at each site, representing 25% crop alleles in each founding population. (a) Glucophosphoisomerase (GPI). (b) Phosphoglucomutase (PGM). (c) Proportion of plants with white petal color (a dominant, crop-derived trait). (d) Proportion of plants with normal pollen fertility (> 70% fertile).

Table 2 Summary of *F*-statistics and *P*-values (in parentheses) from ANOVAS to test for significant differences across populations and years in the frequencies of crop-specific alleles for GPI (glucose-6-phosphate isomerase) and PGM (phosphoglucomutase), white-flowered plants, and plants with > 70% viable pollen (data from Fig. 1; flower color and pollen data from Year 1 were omitted in these analyses; see text)

Trait	Population		Year	
Frequency of crop-specific PGI	$F_{3, 16}$	2.14 (0.1353)	$F_{6, 16}$	8.52 (**0.0003**)
Frequency of crop-specific PGM	$F_{3, 16}$	3.57 (**0.0378**)	$F_{6, 16}$	1.30 (0.3115)
Proportion of white-flowered plants	$F_{3, 24}$	42.06 (**< 0.0001**)	$F_{8, 24}$	4.43 (**0.0021**)
Proportion of plants with > 70% fertile pollen	$F_{3, 15}$	1.53 (0.2025)	$F_{5, 15}$	8.86 (**0.0004**)

P-values of < 0.05 are shown in bold.

hybrids from 'Scarlet Globe' crossed with four wild populations (Table 5; $P > 0.05$). Wild populations had similar pollen fertility ($P > 0.05$), but exhibited significant differences in fecundity ($P = 0.020$) and marginally different numbers of flowers ($P = 0.06$) when population was considered a random factor. Plants from Maine, Michigan and Georgia had similar fecundity, while plants from New York produced significantly more seeds per plant (Table 5).

Within each pair of wild and hybrid groups, hybrids had significantly lower pollen fertility, as expected, and similar fecundity compared with plants from corresponding wild populations (Table 5). Flower number was significantly greater in hybrids than in the corresponding wild plants in all cases except the Michigan population, for which differences were not significant.

Discussion

Persistence of crop alleles in wild radish populations

The three genetic markers from the crop showed very different patterns of persistence (Fig. 1). We expected to see a strong initial decline in frequencies of all crop alleles because F_1 hybrids had delayed flowering that resulted in lower survival to adulthood, 50% lower fecundity per plant, and *c.* 25% lower pollen fertility (Snow *et al.*, 2001). How-

Table 3 Observed frequencies of crop-specific alleles and significant differences from simulations of frequencies expected for genetic drift

	Observed frequency of crop GPI				Observed frequency of crop PGM				Estimated observed frequency of crop petal color allele			
Year	Pop. 1	Pop. 2	Pop. 3	Pop. 4	Pop. 1	Pop. 2	Pop. 3	Pop. 4	Pop. 1	Pop. 2	Pop. 3	Pop. 4
Year 1	0.234	0.234	0.220	0.221	0.234	0.234	0.220	0.221	0.234	0.234	0.220	0.221
Year 3	**0.160***	**0.154***	–	–	**0.186***	**0.144***	–	–	**0.078***	**0.094***	**0.036***	**0.036***
Year 4	0.225	0.213	**0.174***	**0.127***	0.252	**0.096***	0.259	0.266	**0.078***	**0.117***	**0.041***	**0.051***
Year 6	**0.122***	**0.163***	**0.088***	**0.134***	0.223	**0.144***	**0.178***	0.268	**0.093***	**0.088***	**0.023***	**0.029***
Year 7	**0.165***	**0.179***	0.199	**0.154***	**0.198***	**0.114***	0.287	0.248	**0.070***	**0.065***	**0.016***	**0.024***
Year 8	**0.169***	**0.165***	**0.167***	**0.143***	**0.345***	**0.091***	0.235	0.296	**0.064***	**0.064***	**0.019***	**0.022***
Year 10	**0.083***	**0.160***	**0.113***	**0.055***	0.234	**0.075***	0.271	0.171	**0.068***	**0.043***	**0.016***	**0.016***

GPI, glucose-6-phosphate isomerase; PGM, phosphoglucomutase.
Based on data in Fig. 1; frequencies in Year 1 are based on numbers of reproductive adults; observed frequencies for the white petal allele (a dominant trait) were derived from Hardy–Weinberg expectations. Bold values with asterisks indicate significant differences between observed and expected frequencies ($P < 0.05$); see the Materials and Methods for further details. All significant differences represent lower than expected values, except for one case (underlined) which was higher than expected.

Table 4 Population averages (± 1SE) for fitness components of plants from the four F_{10} crop–wild hybrid populations and two wild radish populations, grown in a common garden experiment in Michigan, USA, in 2005

Type	Population	*n*	Number of seeds per plant	Number of flowers per plant	Proportion of fertile pollen grains per plant
Wild	Pellston, MI	43	746 (60)	383 (39)	0.80 (0.022)
	Deer Isle, ME	31	622 (63)	461 (50)	0.80 (0.035)
Hybrid	Population 1	42	858 (105)	516 (66)	0.86 (0.025)
	Population 2	38	746 (74)	525 (70)	0.86 (0.025)
	Population 3	39	581 (44)	405 (35)	0.87 (0.021)
	Population 4	39	922 (100)	556 (80)	0.88* (0.023)

n, number of plants per population. Differences between the two wild populations were not significant ($P > 0.33$); neither were differences among hybrid populations ($P > 0.20$). The only hybrid mean that is significantly different from the wild population means in planned contrasts is indicated with an asterisk ($P < 0.05$; pollen fertility); see text for a description of statistical tests.

Table 5 Population averages (± 1SE) for fitness components of F_2 wild plants and crop–wild F_2 hybrids obtained from hand-pollinations in a glasshouse and grown a common garden in Michigan, USA, in 2005

Type	Population	*N*	Number of seeds per plant	Number of flowers per plant	Proportion of fertile pollen grains per plant
Wild	Pellston, MI	56	949^{b} (82)	291^{ab} (93)	0.82^{a} (0.022)
	Deer Isle, ME	40	940^{b} (87)	347^{a} (21)	0.83^{a} (0.020)
	Leesburg, GA	40	856^{b} (94)	249^{b} (21)	0.82^{a} (0.031)
	Binghamton, NY	24	1309^{a} (124)	333^{a} (29)	0.84^{a} (0.023)
F_2 Hybrid – Group 1	'Cabernet' × MI	39	768^{a} (103)	365^{a} (34)	0.71^{a} (0.035)
	'Cherriette' × MI	39	697^{a} (99)	336^{a} (47)	0.65^{a} (0.030)
	'Red Silk' × MI	24	833^{a} (224)	514^{a} (116)	0.66^{a} (0.032)
	'Scarlet Globe' × MI	39	1069^{a} (206)	446^{a} (79)	0.63^{a} (0.032)
F_2 Hybrid – Group 2	MI × 'Scarlet Globe'	39	1069^{a} (206)	446^{a} (79)	0.63^{a} * (0.032)
	ME × 'Scarlet Globe'	37	1015^{a} (217)	570^{a} * (151)	0.67^{a} * (0.034)
	GA × 'Scarlet Globe'	40	1093^{a} (138)	513^{a} * (69)	0.65^{a} * (0.029)
	NY × 'Scarlet Globe'	38	1172^{a} (161)	609^{a} * (97)	0.69^{a} * (0.028)

Hybrids in Group 1 had one of four cultivars as a paternal parent, and hybrids in Group 2 had one of four wild populations as a maternal parent. Within each wild or hybrid group, differences among populations are indicated with different superscripts ($P < 0.05$). For Hybrid Group 2, significant differences in comparisons between each hybrid and its associated wild parent are indicated with an asterisk ($P < 0.05$).

ever, F_1 hybrids produced more flowers per plant than their wild counterparts when compared in a common garden (Snow *et al.*, 2001), perhaps owing to heterosis. Greater flower production by F_1 plants could boost male fitness (Devlin *et al.*, 1992), but this advantage was likely offset by reductions in other components of hybrid fitness.

Frequencies of white-flowered plants started at 0.50 (owing to dominance) and declined more sharply than

expected for a selectively neutral marker in the second generation (Fig. 1). In subsequent years, frequencies of this crop allele remained lower than expected from simulations of genetic drift (Table 3). In general, white-flowered plants were more than twice as common at Populations 1 and 2 than at Populations 3 and 4. This long-lasting difference among sites is likely related to the disproportionately poor performance of the late-flowering F_1 hybrids and their progeny at Populations 3 and 4. In a separate study, we found that the white petal allele is linked to delayed flowering, a heritable trait (Campbell *et al.*, 2009). This linkage could contribute to the loss of this allele when selection acts against late-flowering plants that failed to set fruit or never bolted.

Declines in frequencies of the white petal allele also could be influenced by factors such as pollinator and herbivore preferences (Stanton *et al.*, 1986; Stanton, 1987; Irwin & Strauss, 2005). We did not obtain data on herbivore preferences. Previous studies show that butterflies and syrphid flies strongly prefer yellow-flowered wild radish plants over white (Kay, 1976; Stanton *et al.*, 1986; Lee & Snow, 1998). Butterflies were infrequent at the experimental populations, while bumblebees, solitary bees, honeybees and syrphid flies were common (A. A. Snow, pers. obs.). In a previous study at UMBS, syrphid flies were the most abundant flower visitors to wild radish plants in experimental arrays (Lee & Snow, 1998). However, the preference of syrphids and other local pollinators for yellow-flowered plants was frequency-dependent and disappeared when crop–wild hybrids were rare. This suggests that pollinator discrimination against white-flowered plants may have declined over time in our hybridized populations, allowing the allele for white petal color to persist throughout the 10 yr of this study.

The GPI crop allele also declined in frequency in all four populations, although it never disappeared, and it did not seem to be linked to the white petal allele (Fig. 1). Several previous studies have shown that variation in GPI is associated with differences in plant survival, fecundity, and pollen performance in other species (reviewed in Lönn *et al.*, 1996; Travers & Mazer, 2001), which suggests that direct effects of this allele on fitness could affect its persistence. In contrast to flower color and GPI, crop-specific PGM alleles (PGM-1 and PGM-3, summed), declined in Population 2 but did not deviate significantly from genetic drift expectations in Populations 3 and 4. For Population 1, frequencies of crop-specific PGM alleles were higher than expected in one year and lower than expected in other two years, with no clear directional trend over time. Reasons for differences among populations and years are not known. In light of the results for flower color and GPI, it is interesting that the crop alleles for PGM did not show much of a decline in three of the four populations (Table 3). This illustrates that patterns of introgression can vary markedly among loci.

Fitness components of introgressed wild radish populations

In the current study and others on *Raphanus* (Panetsos & Baker, 1967; Snow *et al.*, 2001; Campbell *et al.*, 2006), F_1 hybrids from various wild and crop seed sources had only *c.* 60% viable pollen, compared with *c.* 80–95% viable pollen in wild plants, owing to heterozygosity for a reciprocal translocation (Panetsos & Baker, 1967). The speed with which the four experimental populations recovered wild-type pollen fertility in the current study was somewhat surprising given that the seed bank could retard directional selection. However, heterozygosity at both of the two chromosome blocks involved in the reciprocal translocation is expected to decline even in the absence of selection, owing to backcrossing, independent chromosome segregation and recombination during meiosis. In addition, plants with low pollen fertility may have lower reproductive success. We suspect that joint selection for more than one trait could be involved because early flowering in hybrids is genetically correlated with greater pollen fertility (Campbell *et al.*, 2009). This suggests that the wild-type translocation may be linked to early flowering. Thus, the lower fitness of late-flowering F_1 plants could begin to purge the crop version of the translocation from the population. Late flowering was especially common in the F_1 hybrids in Populations 3 and 4, and the faster recovery of pollen fertility at these sites is consistent with this hypothesis.

Although F_1 hybrids produced more flowers and fewer seeds than wild plants (Snow *et al.*, 2001), these early differences were not detected in the common garden experiment with F_{10} plants. Recovery of wild-type fecundity may result from backcrossing with wild plants and selection against fitness-reducing crop traits, such as delayed flowering. In the tenth year, frequencies of the three crop allele markers were generally low to moderate, ranging from < 0.05 to *c.* 0.25, so it is not surprising that the fecundity of these plants was similar to that of the two wild populations.

In a related study at UMBS, we allowed replicated wild vs F_1 crop–wild hybrid radish populations to evolve for four generations and then compared their fecundity in common garden experiments (Campbell *et al.*, 2006). This study differed from the current one in several respects: hybrid populations started with 50% crop alleles rather than 25%; the wild seed source was from Pellston, Michigan, rather than Maine; and we did not monitor crop-specific allozyme frequencies (Campbell *et al.*, 2006). Average pollen fertility increased over time, while the frequency of white-flowered plants remained relatively constant: 70–75% of the plants had white (or pink) flowers in each year of the study, in contrast to the data reported here. In a common garden environment, the fecundity and pollen fertility of F_4 hybrids was somewhat lower than that of wild plants, perhaps because of the greater initial crop allele frequency than

in the current study. However, the relative fitness of hybrids was strongly context-dependent (also reported in wild sunflower, for example, by Mercer *et al.*, 2007). The F_4 hybrids performed much better than wild plants when compared in a parallel common garden in California (Campbell *et al.*, 2006), where weedy hybrid-derived populations have evolved spontaneously (Hegde *et al.*, 2006). Moreover, Ridley & Ellstrand (2009) found that local hybrid-derived populations in California had greater survivorship and fecundity that wild *R. raphanistrum* at several field sites. Together, these results are consistent with other evidence demonstrating that alleles from cultivated radish can persist in hybrid populations, as postulated by Panetsos & Baker (1967) and confirmed by Hegde *et al.* (2006).

Comparisons of wild and hybrid progeny from different source populations

A possible limitation of our long-term study of crop allele persistence is that only one wild population (from Deer Isle, Maine) and one cultivar (Scarlet Globe) were used to create the four replicated populations. However, in the common garden experiments in Michigan, we found no differences between Maine and Michigan populations of *R. raphanistrum* in fecundity, flower number or pollen fertility (Tables 4,5). Although only one population was examined from each state, it was reassuring to find that these two wild populations did not differ in key fitness components. In addition, we did not observe significant differences in fitness components among F_2 progeny from four wild populations crossed with 'Scarlet Globe', or among F_2 progeny from four cultivars crossed with wild plants from Michigan (Table 5). These results suggest that results from the current study may be generalizable, with the caveats that progeny from three of the wild seed sources were not compared in each of their 'home' environments (e.g. in Maine, New York and Georgia), and only one wild population was sampled from each state.

The *relative* fitness of hybrids compared with wild plants was also similar across hybrid groups from different wild populations in most cases (Table 5). The only exception was that crop–wild hybrids from Michigan did not produce significantly more flowers per plant than their wild counterparts (the trend was similar to the other groups). In summary, results from comparisons involving F_2 progeny are consistent with the assumption that hybrids from different wild and cultivar seed sources do not differ greatly in lifetime fecundity or pollen fertility.

Implications for transgene persistence

This study is unique because we report locus-specific changes in crop allele frequencies in introgressed weed populations over a decade. In some cases, crop alleles are not expected to persist, as when initial hybridization rates are low and populations are small enough to be affected by genetic drift, or when the recipient populations simply die out. However, many agricultural weeds have large populations, long-lived seed banks and extensive pollen- and seed-mediated gene flow, all of which can facilitate the spread and persistence of crop alleles (Ellstrand, 2003). Although our study employed only three crop-specific markers, this was sufficient to document both long-term persistence following a single hybridization event and different degrees of introgression among loci. Our findings have implications for understanding the fates of transgenes in wild populations. Clearly, crop alleles can persist for many generations following a single hybridization event, and crop–wild hybrids may recover wild-type fitness in later generations. Thus, beneficial or neutral transgenes that recombine independently of deleterious crop alleles may spread and persist indefinitely. In sunflower, for example, a Bt transgene for insect resistance conferred a large fecundity advantage at one of two field sites, and no fitness costs of this transgene were detected (Snow *et al.*, 2003). In a similar study, Burke & Rieseberg (2003), found no fitness benefits or costs associated with a transgene for white mold resistance in sunflower. These two short-term, common garden studies provide a good starting point for anticipating the fates of introgressing transgenes.

Our current study of hybrid radish populations focuses on variation in rates of crop allele introgression and illustrates the challenge of predicting the extent of transgene introgression without information about fitness effects of closely linked, naturally occurring crop alleles. The combined fitness effects of transgenes and linked crop alleles may vary among transgenic lines, depending on where the transgene is inserted. To overcome this problem, and to reduce the spread of transgenes in free-living populations, some researchers advocate using tandem constructs to link desirable transgenic traits with transgenes for maladaptive traits that are deleterious in weeds or wild relatives but are not harmful to the crop (Gressel, 1999; Papa *et al.*, 2005; Al-Ahmad & Gressel, 2006). This strategy has the potential to greatly diminish unwanted introgression of transgenes, especially if several tightly linked fitness-reducing transgenes are employed, although complete biological containment is probably impractical (USA National Research Council, 2004). As new transgenic traits become available in crops that hybridize with weeds, further research on the interplay between hybrid fitness, linkage to domestication-related traits, and direct fitness effects of transgenes will aid in assessing the extent and possible consequences of gene flow from transgenic crops.

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