RESEARCH PAPER

Persistence of sunflower crop traits and fitness in *Helianthus petiolaris* populations

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ABSTRACT

Transgenic plants have increased interest in the study of crop gene introgression in wild populations. Genes (or transgenes) conferring adaptive advantages persist in introgressed populations, enhancing competitiveness of wild or weedy plants. This represents an ecological risk that could increase problems of weed control. Introgression of cultivar alleles into wild plant populations *via* crop–wild hybridisations is primarily governed by their fitness effect. To evaluate this, we studied the second generation of seven wild–crop interspecific hybrids between weedy *Helianthus petiolaris* and cultivated sunflower, *H. annuus* var. *macrocarpus*. The second generation comprised open-pollinated progeny and backcrosses to the wild parent, mimicking crosses that occur in natural situations. We compared a number of morphological, life history and fitness traits. Multivariate analysis showed that the parental species *H. annuus* and *H. petiolaris* differed in a number of morphological traits, while the second hybrid generation between them was intermediate. Sunflower crop introgression lowered fitness of interspecific hybrids, but fitness parameters tended to recover in the following generation. Relative frequency of wild/weedy and introgressed plants was estimated through four generations, based on male and female parent fitness. In spite of several negative selection coefficients observed in the second generation, introgressed plants could be detected in stands of <100 weedy *H. petiolaris* populations. The rapid recovery of fecundity parameters leads to prediction that any trait conferring an ecological advantage will diffuse into the wild or weedy population, even if F1 hybrids have low fitness.

INTRODUCTION

Modern technologies, including crops with genetic resistance or tolerance to herbicides, have increased interest in the study of crop gene introgression in wild or weedy populations. Gene flow is one of the main evolutionary processes that make possible the emergence of new plant genotypes and species, both domestic and invaders. Crop-wild hybridisation has economic and ecological implications because it can produce the genetic collapse of wild populations or the emergence of new weeds (Ellstrand *et al.* 1999). Genes (or transgenes) conferring adaptive advantages persist in the introgressed populations, enhancing competitiveness of wild or weedy plants; this constitutes an ecological risk that could increase problems of weed control (Linder *et al.* 1998). The scientific information on gene flow from genetically modified (GM) crops to invasive relatives is scarce, even in Argentina, which is the second country in the world in GM crop adoption (James 2009). Although not available to farmers, near 80 sunflower GM varieties have been released under controlled conditions in the last two decades (Cantamutto & Poverene 2007).

Traits of herbicide tolerance, disease resistance or tolerance to abiotic stress (*i.e.,* drought, salinity) have the potential to increase invasiveness under certain environmental conditions (Whitton *et al.* 1997). If tolerance to one or more herbicides were transferred from crop to wild relatives in agricultural environments, wild–crop progeny might inherit tolerance genes, and in a few generations, weed control biotechnology would become ineffective (Clark 2006; Craig *et al.* 2008; Devos *et al.* 2009).

Studies focused on first generation crop–wild hybrid fitness are insufficient as indicators of potential risk of gene/transgene escape because gene movement rate from one population to another depends on fitness of the hybrids and their progeny (Hails & Morley 2005). Even when wild–crop hybrids have lower fitness than their wild parents, further selection for improved fitness could lead to greater invasiveness, and the evolutionary impact will depend on hybrid fitness under natural conditions (Campbell *et al.* 2006; Campbell & Snow 2007). For example, rapid evolution of adaptive weedy traits has been observed in progeny of initially unfit wild–crop *Raphanus* hybrids (Campbell *et al.* 2009a,b).

Experiments with advanced generation hybrids provide better prediction of the persistence of crop alleles in wild populations, since they incorporated natural selection effects (Burke & Arnold 2001). Also, advanced generation hybrid fitness provides a more accurate estimate of the probability of transgene persistence in nature (Linder & Schmitt 1994;

Loureiro *et al.* 2009). Mutations conferring resistance to herbicides, *e.g.* imidazolinones (IMI), could similarly alter plant fitness in a wild population. IMI tolerance has been found in sunflower wild relatives, and in many other families (Al-Khatib *et al.* 1998; Massinga *et al.* 2005). Gene flow from transgenic *Brassica napus* to weedy *B. rapa* can potentially enhance the weediness and/or invasiveness of hybrid and backcross offspring (Al-Ahmad & Gressel 2006). Commercially released transgenes (Simard *et al.* 2006; Fitzjohn *et al.* 2007) and resistance mutations (Kolkman *et al.* 2004; Sala *et al.* 2008) are usually inherited as single Mendelian loci.

Helianthus petiolaris (Asteraceae) is a sunflower relative native to North America with a high invasive potential into Argentine agro-ecosystems. Sixty years after its accidental introduction, it has readily diffused to sandy soils of the semiarid environment in Argentina (Cantamutto *et al.* 2008), forming extended populations of more than 50,000 plants, with a patchy distribution reaching up to 80 plants·m^{-2} (Poverene *et al.* 2008). Geographic distribution of invasive *H. petiolaris* populations strongly overlaps that of the sunflower crop (*Helianthus annuus* var. *macrocarpus*) area. In their centre of origin, both species flower at the same time and share pollinators that favour natural hybridisation through pollen flow (Rieseberg *et al.* 1999).

The domestic sunflower and *H. petiolaris* present low sexual compatibility. In their native habitats, several chromosome rearrangements are responsible for the species isolation (Rieseberg *et al.* 1995). In spite of these reproductive barriers, interspecific hybrids with intermediate morphology are often found in overlapping geographic zones of the invaded environment (Ureta *et al.* 2008). In a previous work, first generation hybridisation events between *H. petiolaris* and domestic sunflower were found in several populations of central Argentina (Gutierrez *et al.* 2010). However, this fact is insufficient to prove crop gene (or transgene) introgression to wild populations. Crop gene persistence into wild or weedy populations or their extinction will depend on fitness of the interspecific hybrids and their progeny, under given environmental conditions (Whitton *et al.* 1997; Linder *et al.* 1998; Cummings *et al.* 2002; Mercer *et al.* 2007). Crop–wild gene flow may benefit weedy *Helianthus* populations (Baack *et al.* 2008), as found in other taxa (Hooftman *et al.* 2005; Letourneau & Hagen 2009). Sunflower crop–wild hybrids can vary greatly from relatively low to comparable fitness with wild plants (Cummings *et al.* 2002). Fitness of crop–wild hybrids is often dependent on environmental factors; stress can increase the relative fitness of such hybrids (Mercer *et al.* 2006, 2007; Dechaine *et al.* 2009).

In the present work, we focus on crop trait persistence in invasive *Helianthus petiolaris* populations of Argentina. (i) How would such traits affect the fitness of crop-introgressed plants? (ii) Would crop traits persist for many generations in wild or weedy populations? The release of new sunflower varieties with advantageous genes (or transgenes) would have minor agro-ecological consequences if crop-introgressed plants were unfit compared to *H. petiolaris* plants, so reducing to very low frequencies in a few generations. If this is not the case, an alert about GM sunflower varieties and a carefully case-to-case study would be necessary before any environmental release.

MATERIALS AND METHODS

Plant material

In a previous work, we reported the occurrence of natural first generation interspecific hybrids between *Helianthus petiolaris* and *H. annuus* var. *macrocarpus* as the pollinator parent (Gutierrez *et al.* 2010). Here, crop gene introgression and fitness effects on *H. petiolaris* populations were studied in the second generation of these interspecific hybrids (IH).

In summer 2002, heads of typical wild plants exposed to crop pollen were sampled at random in 26 *H. petiolaris* populations growing close (<100 m) to sunflower crops in different localities of La Pampa, Buenos Aires and San Luis provinces. The progeny of a subsample of these heads was evaluated at the experimental field of the Agronomy Department, in Bahia Blanca (S 38°41′38″, W 62°14′53″). Nine out of 26 *H. petiolaris* populations produced atypical plants: 13 individuals were demonstrated to be interspecific wild–crop hybrids (IH) among 4374 wild plants (0.3%). Their pollen viability and seed set had very low values compared to the parental species (Gutierrez *et al.* 2010).

Eight IH were sufficiently fertile to obtain seed using their pollen to fertilise *H. petiolaris* plants from the same population. Backcrosses (BC) were obtained from previously bagged *H. petiolaris* heads, applying IH pollen with a cotton brush. Open-pollinated seeds (OP) produced by IH plants without emasculation were collected from these eight IH plants; the remaining five individuals were completely sterile or did not produce enough seed to establish further experimental plots. Complete self-incompatibility of IH plants was demonstrated through the absence of seed when heads (n = 4) were bagged at R3 stage. Male parents of OP were not identified, being comprised of the pollen community of the experimental field. These mating types mimic those occurring naturally in the agro-ecosystems where *H. petiolaris* and *H. annuus* were present and originated IHs. The second generations (BC and OP) derived from a given IH along with *H. petiolaris* from the same geographic location were called a 'family' (Fig. 1).

In total, 342 plants of OP, BC and *H. petiolaris* plants were evaluated in 2004. Seeds were grown in a greenhouse and transplanted to the experimental field at the 4–6 leaf stage, at a density of nine plants·m^{-2}, in a completely random design with three replicates, with supplemental watering and weeding as needed. No agrochemicals were applied. Each experimental unit comprised a family replicate of six individuals of BC and OP progeny of a first generation hybrid and the original *H. petiolaris* population of a given locality (n = 7). Families were identified by the number of the *H. petiolaris* accession: 0102 from Buenos Aires; 0502, 0802, 0902, 4702 from La Pampa; 3202 from San Luis. Two interspecific hybrids were obtained from population 0802 and a complete family was derived from each accession. Family 4702 was excluded because of insufficient BC seed. Domestic sunflower (cv. Dekalb 3900) was included in adjacent lines at the same density as the control.

Trait characterisation

The following morphological traits were recorded in every plant in the experimental field in 2004: plant height, stem diameter, leaf width and length, petiole length, number of
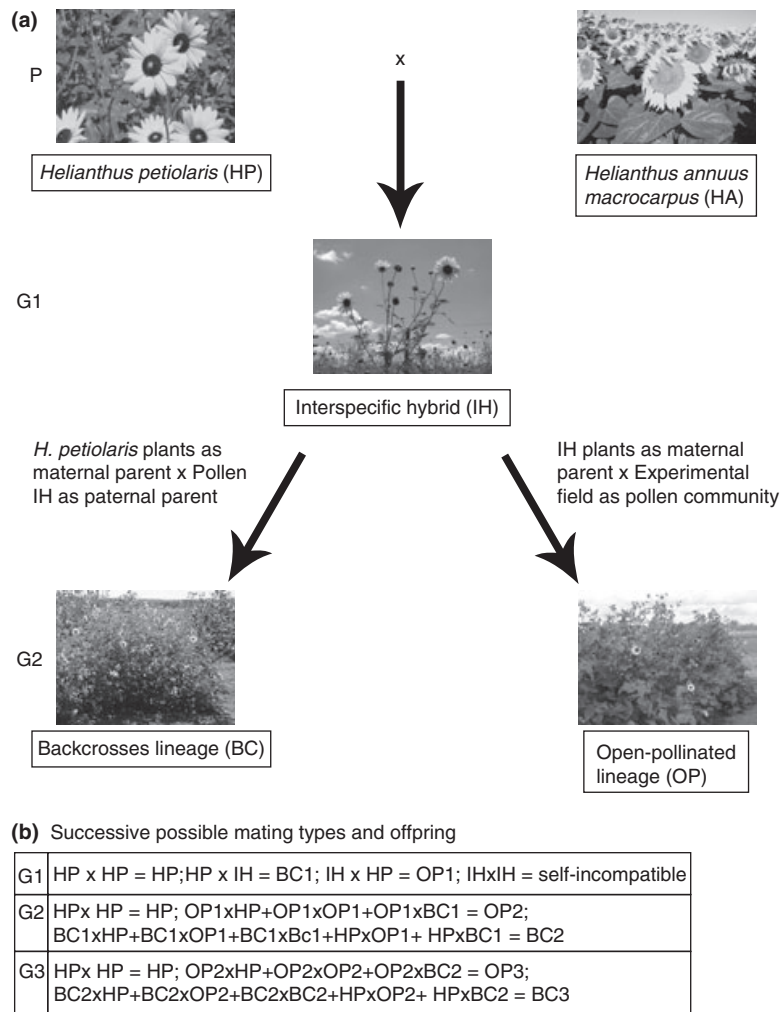
(a)

P — *Helianthus petiolaris* (HP)   x   *Helianthus annuus macrocarpus* (HA)

G1 — Interspecific hybrid (IH)

H. petiolaris plants as maternal parent x Pollen IH as paternal parent

IH plants as maternal parent x Experimental field as pollen community

G2 — Backcrosses lineage (BC)   Open-pollinated lineage (OP)

(b) Successive possible mating types and offpring

G1	HP x HP = HP;HP x IH = BC1; IH x HP = OP1; IHxIH = self-incompatible
G2	HPx HP = HP; OP1xHP+OP1xOP1+OP1xBC1 = OP2; BC1xHP+BC1xOP1+BC1xBc1+HPxOP1+ HPxBC1 = BC2
G3	HPx HP = HP; OP2xHP+OP2xOP2+OP2xBC2 = OP3; BC2xHP+BC2xOP2+BC2xBC2+HPxOP2+ HPxBC2 = BC3

Fig. 1. a: A family formed by the original plant material, *Helianthus petiolaris* (HP) × *H. annuus macrocarpus* (HA) interspecific hybrids (IH) and the derived second generations, backcrosses to the wild parent (BC) and open-pollinated progeny (OP). Eight such families were studied. The pollen donor HA was not analysed as a member of the family. b: Successive possible mating types and offspring.

ligulate flowers, ligule width and length, bract (phyllary) number, bract length and width, and disk diameter. Data were subjected to principal component analysis (bi-plot) from a correlation matrix and compared through the Kruskal–Wallis non-parametric test.

Life-cycle data, days to flowering and days to senescence, and fitness data, survival to flowering, head number, pollen stainability, seed set, germination, dormancy and seed viability, were used to characterise the second generations (BC and OP) of seven IH hybrids and their corresponding wild parent, *H. petiolaris*.

Germination and physical dormancy were studied in an achene pool from the three replicates of each second generation (BC and OP) and the wild parent (*H. petiolaris*) within each family. A hundred achenes were briefly washed in 10% (v/v) sodium hypochlorite, placed in trays on wet paper and kept at 4 °C for 1 week, to break physiological dormancy. Germination was evaluated daily at 20–22 °C over 15 days. In another sample of 100 achenes, physical dormancy was mechanically broken by nicking the end of seeds before stratification. Dormant seed was calculated as the difference in germination between cut and uncut seeds. The mechanical scarification effect on germination within each plant type (*H. petiolaris*, BC, and OP) was compared through the non-parametric Wilcoxon test, using geographical origin as replicate. A confidence interval was obtained for the number of dormant seeds within each group and the variance among groups was analysed by the non-parametric Kruskal–Wallis test. A contingency test was done to evaluate dormancy among populations within each group, comparing uncut germinated seeds and dormant seeds through the Pearson association test, which allowed assessment of the relationship among germination and geographical origin of the analysed population.

Fresh pollen of bagged inflorescences of three *H. petiolaris*, BC and OP plants per experimental unit was collected in Petri dishes. Smears of pollen samples were done on a slide with a stain drop (Alexander 1980). Four hundred pollen grains were counted per slide to estimate pollen viability. With this technique, the protoplasm of non-aborted grains reacts with acid fuchsin, producing a purple colour. Grains stained intense purple were considered viable, while green

grains were considered non-viable. As there is a correlation between pollen stainability and viability, the percentage of non-aborted grains is an approximate estimate of pollen viability. Variance within and among families was analysed with the non-parametric Kruskal–Wallis test.

Reproductive phenology of each plant was estimated as the period (n days) from the date of commencement of flowering (opening of the first inflorescence, R5, Schneiter & Miller 1981) to senescence, which is considered as physiological maturity (R9). Records were made for all the individuals in the experimental field. Normality of data at both stages was checked with the Shapiro–Wilks test and analysed using two-way ANOVA, followed by the Tukey test for means comparison. As data were normally distributed, no transformations before analysis were required.

At R9, the total number of heads per plant was recorded. The seed set per plant was estimated in a sub-sample of three heads per plot of each group (*H. petiolaris*, BC and OP) previously bagged to avoid seed loss through shattering. Filled and empty achenes per head were visually screened and seed production was calculated as a product of head number per plant and filled fruits per head. Data were analysed with the Kruskal–Wallis test.

To examine among-trait relationships, Pearson correlation coefficients (r^2) were generated for all trait combinations for *H. petiolaris*, BC and OP populations. Differences in regression coefficients between groups were tested using ANCOVA (Campbell *et al.* 2009a) to determine whether the strength of phenotypic selection differed between wild and hybrid populations. Data from the three plant types (*H. petiolaris*, BC and OP) were combined and analysed with a model that included continuous terms for 14 phenotypic traits, a categorical term coding for plant type and phenotypic traits–plant type terms. Seed production per plant was used to calculate relative fitness, which was the dependent variable. Fitness and standardised phenotypic traits were relativised separately for each plant type. We compared the slope of the fitness–phenotypic trait relationship between the three populations using a 1-*df* contrast on the phenotypic trait–plant type term. Significance levels were assigned using the F-distribution. All analyses were completed using InfoStat (2006).

Assessment of introgression dynamics

A conservative estimation of crop introgression was assessed considering the fitness parameters measured under the growing conditions of the experimental field, where there was no competition for water or space. For this estimate, the families were considered as replicates. The observed frequency of IH plants recorded by Gutierrez *et al.* (2010) was used for the first generation of six families that produced enough seed (G1, Fig. 1). Frequency of each plant type (*H. petiolaris*, OP and BC) in the following generations, G2 and G3, was calculated as the mating probability corrected by the relative fitness of both parents (Fig. 1). The female parent relative fitness was estimated as seed production, and the relative fitness of the male parent was estimated as pollen stainability, where *H. petiolaris* fitness = 1. This procedure was used to estimate the frequency of introgressed plants within two lineages originated in an IH plant as male (BCn) and female (OPn) for each family. To investigate differences in biotype frequencies between families at the G3 genera-

tion, we calculated the mating probabilities for each replicate in the G2 experimental field. This allowed three replicates for each biotype × family combination, which were analysed through ANOVA after $\sqrt{x + 1}$ transformation. To estimate the fourth generation (G4), plant frequency was calculated taking into account the mating probability and considering that the introgressed plants (OP and BC) maintained the fitness value of the G3 plants. For each generation, a mean frequency of each biotype and its 95% confidence interval was calculated, based on six families. For each family, a minimum stand was calculated as the least number of individuals that would allow detection of an introgressed individual produced by either of two lineages.

RESULTS

Morphological characterisation

The second generations of the interspecific hybrids between *H. petiolaris* and domestic sunflower showed an overall intermediate morphology between the two parental taxa (Fig. 2). Many, but not all, BC plants were similar to the female parental *H. petiolaris*. The OP progeny of hybrid plants and other BC individuals were scattered among wild and cultivated plant types. A higher variability was observed among OP progeny compared with backcrosses.

Nine metric morphological traits distinguished BC and OP individuals from *H. petiolaris* and domestic sunflower (Table 1). Highly significant differences were found between OP progeny of the first generation hybrids, backcrosses and cultivated sunflower, except for bract (phyllary) number. No differences for ligule length and width were found between *H. petiolaris* and backcrosses.

Life history and fitness traits

H. petiolaris and OP offspring differed in eight out of 10 life history and fertility parameters, while only four characters distinguished the wild parent from the BC, and seven differed between BC and OP plants (Table 2).

There were no differences in survival among the wild parent and the second hybrid generations. The OP generation began flowering 3 days earlier and the life cycle ended 4 days earlier than in the wild parent. The BC generation, where IH was the male parent, did not differ from *H. petiolaris* in life cycle traits. The second hybrid generations OP and BC had significantly lower head number than the wild parent *H. petiolaris*. Pollen stainability and seed set had marked differences among the two hybrid generations and *H. petiolaris*; OP having <15% pollen stainability and seed set. BC plants also differed significantly, but only by 10% less than *H. petiolaris*. As a consequence, seed production per plant was significantly lower in the second hybrid generations. OP produced <30% seed compared to *H. petiolaris*, while BC produced more than 80% seed than OP.

Seed germination in the second hybrid generations tended to be higher than in the wild parent; OP significantly differed from *H. petiolaris*. BC germination did not differ from OP and *H. petiolaris*. Physical scarification increased achene germination in the second generations and the same in the wild female parent. Comparison did not show differences in dormancy degree of *H. petiolaris* and BC, but there were differences between these two plant types and the OP progeny (Table 2).
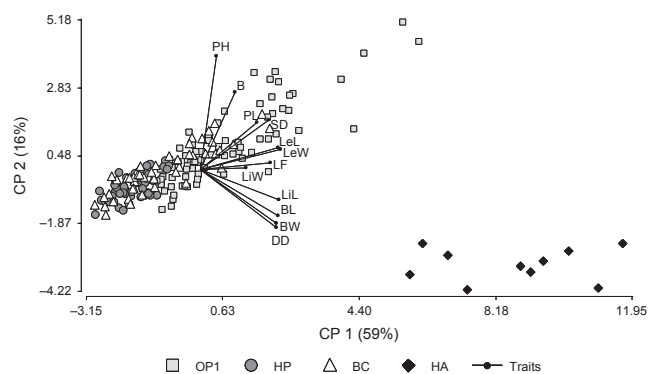
Fig. 2. Principal component analysis (bi-plot) based on 12 metric morphological traits. Each symbol represents an individual of *H. petiolaris* HP, BC, OP and domesticated sunflower, HA. For bi-plot references see Table 1. N = 342 plants.

Highly significant differences were detected among families for life-cycle stages, significant differences for the family × type interaction at the beginning of flowering and highly significant differences for the same interaction at senescence. Regarding head number, differences were also found between localities for BC and OP, while there were no differences between *H. petiolaris* populations for head number. Seed set only showed significant differences between localities for BC. There were no differences among localities for pollen stainability. These results indicated considerable variation among families.

To determine if trait evolution could be influenced by correlated selection, we estimated phenotypic correlations among traits for each plant type. For BC there were 46 significant correlations among traits (Table 3); disk diameter was positively correlated with all the traits, while head number was negatively correlated with ligule width and bract number, suggesting that crop traits determine a lower fitness. For OP there were 29 significant correlations, all of them positive. Taller plants had tougher stems, bigger leaves and heads (as indicated by bract number and width and disk diameter) showing a strong influence of crop traits, but negative correlations with head number were not significant. Only five traits were significantly correlated in *H. petiolaris* (data not shown).

Table 1. Morphological traits (means ± SD) of second generations of interspecific wild–crop hybrids (IH) and their parents. *Helianthus petiolaris* (HP), backcrosses (BC), open-pollinated progeny (OP) and domestic sunflower (HA). Variable units are cm, except for head and bract number.

variable & symbol		wild parent	second IH generations		crop
		HP	BC	OP	HA
plant height	PH	123.6 ± 6.8 a	132.1 ± 14.3 b	144.8 ± 20.7 c	168.3 ± 10 d
stem diameter	SD	0.9 ± 0.2 a	1.08 ± 0.3 b	1.6 ± 0.6 c	2.3 ± 0.5 d
leaf width	LeW	5.5 ± 1.1 a	7.1 ± 2.7 b	11.9 ± 5.4 c	25.6 ± 4.6 d
leaf length	LeL	8.2 ± 1.3 a	9.8 ± 2.7 b	13.7 ± 5 c	25 ± 2.8 d
petiole length	PL	8.3 ± 1.5 a	9.8 ± 2.6 b	11.7 ± 3.8 c	15.1 ± 3.3 d
ligule number	LN	20.8 ± 3.4 a	23.1 ± 4.2 b	25.5 ± 5 c	41.2 ± 8.5 d
ligule width	LiW	1.1 ± 0.3 a	1.1 ± 0.3 a	1.4 ± 0.3 b	1.9 ± 0.1 c
ligule length	LiL	2.8 ± 0.5 a	3 ± 0.7 a	4 ± 0.6 b	8.7 ± 0.7 c
bract number	BN	30.2 ± 3.7 a	32.6 ± 4.5 b	33.4 ± 6.1 b	34.7 ± 3.8 b
bract length	BL	1.3 ± 0.2 a	1.6 ± 0.3 b	2 ± 0.5 c	7.1 ± 1 d
bract width	BW	0.4 ± 0.1 a	0.5 ± 0.1 b	0.6 ± 0.2 c	3.6 ± 0.5 d
disk diameter	DD	2.3 ± 0.2 a	2.7 ± 0.5 b	3.3 ± 0.7 c	19 ± 3.7 d

Means followed by different letters indicate significant differences in Kruskal–Wallis test (P < 0.05). N = 372 (108 HP, 108 BC, 126 OP, 30 HA).

Table 2. Life history and fertility parameters of second generation interspecific wild–crop hybrids, backcrosses (BC), open-pollinated progeny (OP) and their wild parent *Helianthus petiolaris* (HP).

variables	wild parent	second IH generation	
	HP	BC	OP
survival to R5 (%)	94.4 ± 1 a	94.4 ± 0.9 a	99.2 ± 0.4 a
days to R5 (d)	33.8 ± 1.4 a	33.6 ± 1.8 a	30.5 ± 1.7 b
days to R9 (d)	121.2 ± 3.6 a	120.5 ± 3.4 a	113.5 ± 4.3 b
pollen stainability (%)	98 ± 3.4 a	91 ± 0,99 b	11 ± 1.1 c
head number (n/pl)	125.0 ± 1.2 a	114.3 ± 1.9 b	102.8 ± 4.3 c
seed set (%)	95 ± 1 a	82 ± 3.1 b	14 ± 5.9 c
seed production (n/pl)	11242.6 ± 2054.4 a	9892.9 ± 2371.9 b	3132.9 ± 2082.8 c
germination (%)	37.7 ± 7.8 a	41.2 ± 9.4 ab	57.0 ± 17.3 b
dormancy (%)	41.2 ± 8.7 a	36.7 ± 11.4 a	23.1 ± 12.1 b
seed viability (%)	78.8 ± 13 a	77.8 ± 13.7 a	80.3 ± 11.5 a

Mean ± SD. Means followed by different letters indicate significant differences in ANOVA or Kruskal–Wallis test (P < 0.05). N = 342 (108 HP, 108 BC, 126 OP).

Slender stems were selected among BC plants ($\beta = -0.41$; Table 4). In turn, this trait was positively correlated with leaf, bract and ligule size, ligule number and disk diameter (Table 3). Also, narrow ligules were selected ($\beta = -0.22$), a trait positively related with plant height, leaf size, bract size and number, and disk number. These results indicate that wild/weedy traits have higher fitness among BC plants. Significant regressions for relative fitness as a function of standardised trait values were found for BC, but not for OP (Table 4). There was no significant selection for disk diameter in BC or OP, however BCβ and OPβ coefficients indicate that seed production decreased in larger heads, a crop-related trait (Table 4). There were several significant differences in β-values among plant types, indicating complex patterns of selection on these traits.

Introgression dynamics

According to the estimate based on mating frequency and relative fitness, with the IH lineage as female parent (OPn),

frequency exponentially decreased in successive generations after the hybridisation event by $<3 \times 10^{-4}$ in the third generation. On the other hand, lineage of IH as male parent (BCn) increased in frequency, although at a substantially smaller absolute rate (Fig. 3). Family 3202 showed the higher fitness, reaching 8.8×10^{-3} frequency of introgressed plants in the third generation, while family 0502 showed the lowest fitness, reaching only 1.2×10^{-3}. In all cases, the highest contribution of introgressed plants since the hybridisation event came from the BC lineage. In G3, the frequency of both introgressed biotypes was significantly different among all the families (data not shown, but available upon request from the authors). Considering the two lineages (BC plus OP) in each generation, the least stand size necessary to find one introgressed individual was 60 individuals in the G1, and its frequency decreased to a similar G1 value in G4 (Fig. 4). For the family showing the least fitness, the stand size necessary to detect an introgressed plant was three times larger, and by G4 was still two times larger. So IH lineages did not show the same relative contribution in the different families exam-

Table 3. Pearson correlation coefficients among traits in backcrosses (bottom diagonal, N = 108) and open-pollinated (top diagonal, N = 126) populations.

	PH	SD	LeS	PL	LN	LiW	LiL	BN	BL	BW	DD	HN
PH		**0.67**	**0.46**	**0.47**	0.32	0.07	0.17	**0.38**	0.22	**0.46**	**0.44**	0.06
SD	**0.66**		**0.73**	**0.69**	**0.40**	0.03	0.24	**0.46**	0.34	**0.60**	**0.53**	1.3E-03
LeS	**0.71**	**0.50**		**0.64**	0.29	0.15	0.27	**0.35**	**0.58**	**0.69**	**0.67**	0.10
PL	0.20	0.37	0.46		**0.37**	0.09	0.32	**0.42**	0.31	**0.36**	0.34	−0.11
LN	**0.55**	**0.62**	**0.54**	0.43		−0.03	0.17	**0.62**	0.13	**0.38**	**0.55**	−0.23
LiW	**0.46**	**0.41**	**0.62**	0.30	0.37		**0.48**	−0.18	0.09	0.11	0.17	0.11
LiL	**0.66**	**0.61**	**0.71**	0.49	0.56	**0.73**		0.12	0.19	0.30	0.33	−0.05
BN	**0.43**	0.32	**0.46**	0.33	**0.77**	**0.42**	**0.50**		0.19	**0.48**	**0.50**	−0.24
BL	**0.63**	**0.55**	**0.60**	0.27	**0.42**	**0.42**	**0.70**	0.31		**0.58**	**0.58**	0.18
BW	**0.75**	**0.64**	**0.73**	0.37	**0.52**	**0.41**	**0.55**	0.35	**0.52**		**0.77**	−0.02
DD	**0.63**	**0.52**	**0.81**	0.44	**0.53**	**0.67**	**0.85**	**0.54**	**0.64**	**0.62**		−0.09
HN	−0.26	−0.16	−0.36	−0.06	−0.30	**−0.38**	−0.30	**−0.50**	−0.31	−0.21	−0.35	

Significant correlations are given in bold type. Symbols are as in Tables 1 and 2. Leaf size (LeS) results from LeLx LeW.

Table 4. Phenotypic selection analysis. Estimates of linear selection of each trait on seed production. Selection gradients that differ significantly among plant types are indicated by the P-value (F-statistic).

	selection coefficients			significance of β comparisons		
trait	HP β	BC β	OP β	P-value HP–BC	P-value HP–OP	P-value BC–OP
plant height	0.01	0.09	−0.01	0.36	0.3646	**0.0057**
stem diameter	−0.13	**−0.41**	0.07	0.3446	0.107	**0.0068**
leaf width	−0.55	−0.45	0.56	0.3574	**0.0020**	0.4126
leaf length	−1.16	1.02	−0.09	**<0.0001**	**<0.0001**	**<0.0001**
leaf size	3.04	−0.92	−0.27	**<0.0001**	**<0.0001**	**0.00056**
leaf shape	0.23	−0.31	0.12	**<0.0001**	0.9795	**<0.0001**
petiole length	0.16	−0.13	−0.11	0.0766	0.2606	0.8881
ligulate flowers	−0.03	0.05	−0.10	**<0.0001**	0.5562	0.1260
ligule width	−0.18	**−0.22**	3.4E-03	0.8287	0.0268	0.0509
ligule length	0.09	0.33	0.04	0.1902	0.4368	**0.0101**
bract number	−0.20	−0.10	0.05	0.3652	0.0068	0.0242
bract length	−0.19	−0.03	−0.03	0.1772	0.1121	1
bract width	−0.06	−0.16	−0.05	0.4492	0.9094	0.2099
disk diameter	**0.24**	−0.11	−0.03	0.0142	**0.0063**	0.0361

Significant values at P < 0.001 are in bold type. N = 342 (108 HP, 108 BC, 126 OP).

ined. Both with the lowest and the highest fitness, the least stand size necessary to obtain one introgressed plant increased by three-fold in the following generation after the hybridisation event.

DISCUSSION

Introgression of crop genes in *H. petiolaris* could have played a major role in the rapid establishment of this species in Argentina. Early studies reported a low rate of introgression between *H. petiolaris* and *H. annuus*, with a frequency of F1 crop–wild hybrid formation similar to that reported for natural hybrid zones, between two wild species (Rieseberg *et al.* 1999). Nevertheless, this provides ample opportunity for recombination between the two species, and fairly high rates of complex backcross genotypes have been detected (Yatabe *et al.* 2007; Strasburg & Rieseberg 2008; Kane *et al.* 2009). Agriculture and other human activities can increase gene flow between populations (Kane *et al.* 2009).

Crop–wild hybridisation may promote invasive success by enhancing growth and competitive ability (Ellstrand & Schierenbeck 2000; Snow *et al.* 2001; Gressel 2005). Also, they may create genotypes with the potential to displace parental taxa in new environments (Campbell *et al.* 2006). Invasive success could be increased if new hybrid gene combinations enhance grow and competitive ability, as in certain annual *Helianthus* in novel environments in North America (Rieseberg *et al.* 2007). Geographically isolated populations might evolve in parallel; as *H. petiolaris* has successfully colonised extended areas in this part of South America (Poverene *et al.* 2008), it is likely that populations in Argentina harbour crop genes that could increase their invasiveness.

Our study of sunflower crop trait introgression in *H. petiolaris* populations, followed by modification of fitness components, focused on the second hybrid generation following a wild–crop hybridisation event. The interspecific wild–crop hybridisations took place in the area of a sunflower crop, although for practical purposes their frequency was estimated
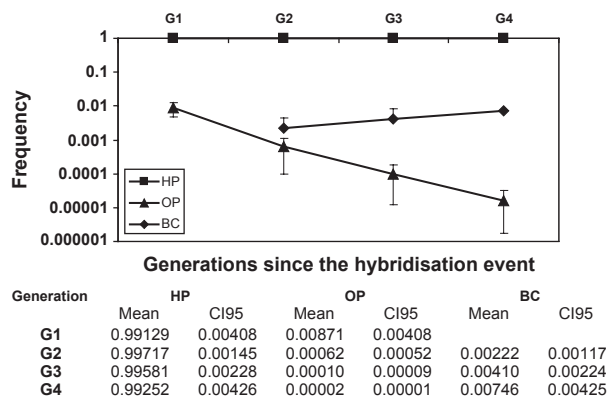

Fig. 4. The least number of plants in a weedy stand of *Helianthus petiolaris* required to detect a crop-introgressed plant, with the lowest fitness (MIN) and the highest fitness (MAX) estimated through six site situations.

in an experimental field (Gutierrez *et al.* 2010). The second hybrid generations were re-created in the experimental field, allowing the first generation hybrids (IH) to produce offspring, using the interspecific hybrid as pollinator of *H. petiolaris* plants (BC), or as female parent under open-pollination conditions (OP). Both types of cross are likely to occur in the agricultural ecosystem. Our main interest was to determine if crop traits persisted and affected plant fitness. As IH came from different localities, geographically scattered through three provinces, a second question was whether the effects were comparable among hybridisation events.

Multivariate analysis showed that the parental species *H. annuus* var. *macrocarpus* and *H. petiolaris* differed in 12 morphological traits, while the second hybrid generations between these two species differed in at least 10 traits. Interspecific hybrid progeny were intermediate when they came from open pollination, or resembled *H. petiolaris* when they came from backcrosses to the wild population. OP origin as crop backcrosses was unlikely as none of these progeny resembled crop plants (Fig. 2). Some BC individuals that did not differ from *H. petiolaris* plants could be the product of selfing because of a mentor effect (Desrochers & Rieseberg 1998). It is likely that at least one part of the BC plants was *H. petiolaris*, a product of *H. annuus* pollen-induced selfing in an otherwise self-incompatible species. However, most were true BC plants according to the morphological analysis. Introgression occurs even when individuals can show no noticeable intermediate or hybrid-like morphologies because gene flow has more effect on some loci whereas other loci remain differentiated (Kane *et al.* 2009). Morphological identification of individuals of hybrid origin in wild populations becomes more difficult beyond the first generation after hybridisation (Heiser 1947). Introgression of crop genes was demonstrated in individuals collected in *H. petiolaris* populations growing close to sunflower crops in North America through molecular markers, however none of them showed morphological evidence of hybridisation (Rieseberg *et al.* 1999). Allele introgression through hybridisation relies more on its effects on fitness than in the rate of gene flow (Burke & Rieseberg 2003). Some crop traits, *i.e.,* lack of dormancy, could have negative effects on wild or weedy plant fitness

Fig. 3. Estimated relative frequencies (means and 95% confidence interval, vertical bars, CI95 in table) of *Helianthus petiolaris* (HP), backcrosses (BC), and open-pollinated plants (OP) in four generations of progeny (Gn) of interspecific hybrids (IH) following a natural wild–crop hybridisation event in six weedy populations. In G1 OP = IH plants. Estimates were made based on data from two field seasons, with N = 4374 in G1, and N = 468 in G2.
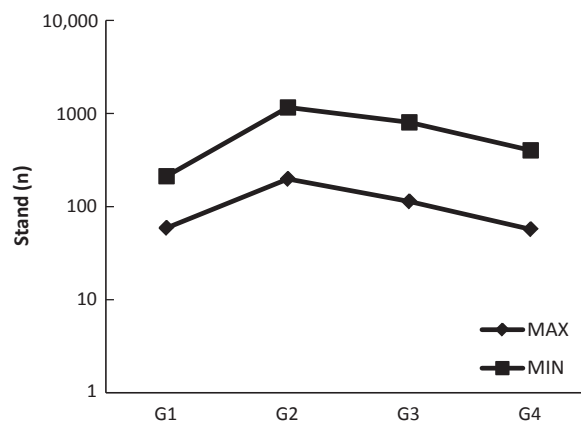
Generation	HP		OP		BC	
	Mean	CI95	Mean	CI95	Mean	CI95
G1	0.99129	0.00408	0.00871	0.00408		
G2	0.99717	0.00145	0.00062	0.00052	0.00222	0.00117
G3	0.99581	0.00228	0.00010	0.00009	0.00410	0.00224
G4	0.99252	0.00426	0.00002	0.00001	0.00746	0.00425

(Snow *et al.* 1998; Stewart *et al.* 2003; Mercer *et al.* 2006). Here, dormancy was higher in the wild pure species compared to the interspecific progeny. Reduced dormancy in hybrids was also found in crosses between wild and cultivated *H. annuus* (Snow *et al.* 1998) and between *Brassica rapa* and rapeseed, *B. napus* (Allainguillaume *et al.* 2006). Soil seed banks are a source of genetic material, and dormancy can be important for the introduction of novel genetic diversity in invasive plant species (Schierenbeck & Ellstrand 2009). The same pattern accounted for life-history traits and germination, although no differences were observed in survival, probably because seedlings were transplanted to the experimental field without competence, so avoiding self-thinning. On the other hand, pollen stainability, seed set and head number differed markedly among *H. petiolaris*, BC and OP. *H. petiolaris* and *H. annuus* differ in several chromosome rearrangements (Rieseberg *et al.* 1995; Burke *et al.* 2004) that may explain the first two possibilities; and these traits rapidly recover in the following generations (Heiser 1947; Rieseberg *et al.* 1996). Head number differed among the three plant types, although it did not show a marked recovery in the second generation following hybridisation, probably because it is a branching associated trait. Genetic control of branching is related to dominant alleles in wild species and to recessive factors in sunflower commercial varieties (Miller & Fick 1997). Crop genes promote lower branching and lower head number in crop–wild hybrids. Natural selection seems to be the main cause of fitness trait recovery, as also observed in *Raphanus* (Campbell *et al.* 2006). When plants of hybrid advanced generations grow in competition with pure wild plants, hybrid fitness increases, promoting introgression of crop genes into wild plants (Campbell & Snow 2007).

A characteristic trait of cultivated sunflower is early uniform flowering and a short definite life cycle compared to the later flowering and long indefinite life cycle of the wild species. Here, OP hybrid progeny began flowering before the pure wild plants and reached senescence earlier, but showed more than 97% overlap in flowering time. On the other hand, BC had a life cycle similar to *H. petiolaris*, ensuring mating opportunity. Variation among families for some of the studied traits was attributed to different parent genetic background. Female parents could differ as a consequence of natural selection in the original geographical sites. This also might be explained through differences inherent in the male parent, which was a commercial sunflower hybrid of unknown pedigree.

The negative correlation between crop traits and fitness, represented by seed production, persisted in the following generation after hybridisation. Selection could be masked by indirect selection through some correlated characters, as no significant regressions were found for OP. Otherwise, the relatively low number of obtained plants did not allow expression of all the expected variability, as OP produced less seed than the other biotypes. Even if there were not many significant relationships between morphological traits and fitness, plant height, leaf size, bract size and head diameter were negatively selected, indicating a lower fitness for crop traits. The results indicate that even wild/weedy traits of higher fitness BC plants contributed offspring to the following generations, which resemble the wild phenotype. In this way, neutral crop traits could persist within weedy *H. petiolaris* populations.

For example, populations could retain herbicide tolerance, a neutral trait in the wild if no fitness costs were associated. Head diameter was negatively related to seed production in BC and OP biotypes, but not in *H. petiolaris* plants. This difference was likely due to chromosome incompatibility among *H. annuus* and *H. petiolaris*, which determines the high number of aborted seeds. Although crop introgression reduced fitness in weedy plants and this could limit population dynamics, introgressed plant frequency slightly increased in the following generations. As morphological traits were measured in the experimental field where no competition for water or space was operating, one caveat is how hybrid fitness might change under stressful environments. In *H. annuus*, Mercer *et al.* (2007) found a fitness increase of wild–crop hybrids under competition in stress conditions. Although those hybrids differ because there is no chromosome incompatibility between them, *H. petiolaris* wild–crop hybrids could also change their fitness under stressful conditions. This should be evaluated in future experiments.

High density stands of natural populations of *H. petiolaris* plants could yield more than 7.4×10^4 achenes per·m^{-2}. Considering the OP seed production ratio (Table 2), of more than 1.1×10^4 seeds per·m^{-2}, this is enough to ensure the persistence of introgressed crop genes in the soil seed bank, even with a high mortality rate. To assess the rate of introgressed *versus* non-introgressed (wild or weedy) *H. petiolaris* plants, the probability of mating types was combined with the relative fitness of each plant type. While OP progeny radically decreased in the successive generations, BC progeny slightly increased, ensuring crop gene maintenance and perhaps accumulation in the weedy populations (Fig. 3). The six families showed the same tendency within a fitness range attributable to fertility and/or ecology selection in the original environment of each population. Depending on fitness, the frequency of introgressed *versus* non-introgressed plants could vary by 10-fold (Fig. 4). In spite of several negative selection coefficients observed in BC and OP generations, introgressed plants might be detected in stands of <100–1000 weedy *H. petiolaris* populations.

In most cases it is expected that domestication traits will dramatically decrease fitness of wild–crop hybrids (Snow *et al.* 1998; Cummings *et al.* 2002; Mercer *et al.* 2007). In our experiments, sunflower crop introgression effectively lowered fitness of hybrid plants. However, the rapid recovery of fecundity parameters following hybridisation allows prediction that any trait conferring an ecological advantage, *i.e.,* herbicide tolerance, transgenic insect or disease resistance, will diffuse into the wild population even if F1 hybrids have a low fitness. Imidazolinone tolerance, which characterises Clearfield® sunflower varieties, can be transferred to *H. petiolaris* (Massinga *et al.* 2005). A Bt transgene enhanced fitness in experimental plots of wild *H. annuus* (Snow *et al.* 2003). Transference of 'ecologically risky' traits might be avoided if appropriate control strategies are implemented. Mitigation technologies couple the herbicide tolerance genes or transgenes with mitigation genes that are neutral or beneficial to the crop but render unfit wild–crop hybrids. A tandem construct, including a primary herbicide-resistant gene and a dwarfing mitigator gene in *Brassica napus*, were effective to preclude the risks of gene establishment in the related weed *B. rapa* and its backcross progeny (Al-Ahmad & Gressel

2006). Mitigation genes confer traits such as non-shattering, dwarfism, no secondary dormancy and herbicide sensitivity (Gressel & Valverde 2009) that would be suitable for preventing mutation or transgene escapes in sunflower.

The present work showed the persistence of crop traits in *H. petiolaris* populations through the female lineage of wild–crop interspecific hybrids. Despite their low fitness, the seed number contributing to the following generation is very high, more than 300-fold the average plant density observed in natural populations (Poverene *et al.* 2008). The male hybrid lineage also showed clear evidence of crop introgression and contributed more that 1000-fold the average plant density. Even though an extreme proportion as two-thirds of the BC progeny came from *H. petiolaris* selfing, the introgressed propagules would be sufficient to ensure pervasiveness of crop traits in wild or weedy populations. As *H. petiolaris* natural populations show high reproductive ability, in spite of the adverse effects of crop trait introgression on fitness at individual level, this is not enough to neglect the risk of ecological impact.

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