Rapid evolution of weedy traits during sunflower de-domestication: the importance of hybridization and standing genetic variation

Running tittle: Rapid evolution of weedy traits in sunflower

Fernando Hernández[1,2], Roman B. Vercellino[1,2], Claudio Pandolfo[1,2], Jennifer R. Mandel[3], Alejandro Presotto[1,2]

[1]Departamento de Agronomía, Universidad Nacional del Sur (UNS), San Andrés 800, 8000, Bahía Blanca, Argentina
[2]CERZOS, Universidad Nacional del Sur (UNS)-CONICET, Camino La Carrindanga Km 7, 8000, Bahía Blanca, Argentina
[3]Department of Biological Sciences, Center for Biodiversity Research, University of Memphis, Memphis, TN 38152

e-mail addresses (in order):
fhernandez@cerzos-conicet.gob.ar
rbvercellino@cerzos-conicet.gob.ar
cpandolfo@cerzos-conicet.gob.ar
jmandel@memphis.edu
apresotto@uns.edu.ar

Corresponding author address: Departamento de Agronomía, Universidad Nacional del Sur, San Andrés 800, 8000, Bahía Blanca. Phone: 54 291 4595102, fax: 54 291 4595127, e-mail: fhernandez@cerzos-conicet.gob.ar

ABSTRACT

Hybridization between crops and their wild relatives may promote the evolution of de-domesticated (feral) weeds. Wild sunflower is typically found in ruderal environments, but crop-wild hybridization may facilitate the evolution of weedy biotypes. Using one crop-specific mitochondrial marker (CMS-PET1) and 14 nuclear SSR markers, we studied the origin and genetic diversity of BRW, a recently discovered weedy biotype. Then, using a resurrection approach, we tested for rapid evolution of weedy traits (seed dormancy, herbicide resistance, and competitive ability) by sampling weedy and wild biotypes 10 years apart (2007 and 2017). All the weedy plants present the CMS-PET1 cytotype, confirming their feral origin. At the nuclear markers, BRW showed higher genetic diversity than the cultivated lines, as high genetic diversity as the most diverse wild biotypes, and low differentiation with one wild biotype, suggesting that wild hybridization increased the genetic diversity of the feral BRW. Regarding weedy trait evolution, we found support for rapid evolution towards higher seed dormancy, but not for higher competitive ability or herbicide resistance. Standing genetic variation probably facilitated the evolution of seed dormancy and limited the evolution of herbicide resistance, as no resistant alleles were found in the ancestral biotype. Our results demonstrate that natural crop-wild hybrids can evolve quickly in farmers' fields, leading to the establishment of weedy biotypes of cultivated origin. Although herbicide resistance did not evolve in BRW, management strategies aimed at preventing the evolution of resistance should be a priority in order to avoid the emergence and spread of herbicide resistant biotypes in Argentina.

1. INTRODUCTION

Since the origins of agriculture, farmers have grown domesticated plants in artificially benign environments, characterized by frequent disturbance, high nutrient and water availability, artificially controlled pests, and monoculture or short-term rotations (Clement, 2014). These modified environments are beneficial for maximizing crop yields, but they also represent novel niches for opportunistic plant species, namely agricultural weeds (Baker, 1974; De Wet and Harlan, 1975). Here, we define weeds as those biotypes that interfere with agriculture, regardless of their wild, cultivated, or mixed origin, and wild biotypes as those that grow and reproduce away from the agriculture environments (outside agricultural plots), whether native or not (Ellstrand et al., 2010). Weeds compete with crops for resources, such as light, nutrients, and water, representing a major cause of crop losses worldwide, estimated at 34 % reduction in crop productivity (Oerke, 2006).

Agricultural environments exert high selection pressures on weeds, and weeds often respond by evolving traits that increase fitness in these environments (Baker, 1974; Huang et al., 2017). Among these traits, seed dormancy and herbicide resistance are among the most important for the successful establishment of weeds. Seed dormancy plays a crucial ecological role, contributing to avoidance of out of season germination (Presotto et al., 2020; Hernández et al., 2021), and persistence in soil seed banks (Darmency et al., 2017; Pipatpongpinyo et al., 2020; Presotto et al., 2020). On the other hand, the high killing rates associated with herbicide applications exert strong selection pressure for herbicide resistance (Darmency et al., 2017; Kreiner et al., 2018; Tranel and Wright, 2002).

Herbicides inhibiting the enzyme acetohydroxy acid synthase (AHAS) are among the most widely used herbicides worldwide. Its simple genetic architecture, and the discovery of alleles confering resistance in several cultivated species, allowed the development of the non-transgenic Clearfield® technology (Tan et al., 2005). However, the drawback of this group of herbicides is their propensity to generate resistant biotypes (Tranel and Wright, 2002). AHAS-inhibiting herbicide resistant biotypes have been reported in at least 166 species worldwide (Tranel et al., 2021), including weedy sunflower (Al-Khatib *et al.* 1998; White *et al.* 2002; Tranel et al., 2021).

Weedy traits may evolve by selection on the standing genetic variation (Huang et al., 2017), new mutations (Al-Khatib et al., 1998; Vercellino et al., 2018), or by adaptive introgression (Le Corre et al., 2020; Pandolfo et al., 2018; Vigueira et al., 2019). Evolution from the standing

genetic variation is likely to be much faster than from new mutations (Kreiner et al., 2018), given that pre-existing variants are readily available for selection to act on, and at a higher frequency than new mutations. On the other hand, when no genetic variation is present, adaptive introgresion can quickly provide adaptive variants in populations under selection (Le Corre et al., 2020; Pandolfo et al., 2018; Vigueira et al., 2019), though source and recipient populations must be cross-compatible and coincide in space and time (Ellstrand et al., 2010).

Ferality or de-domestication is an evolutionary process by which domesticated species escape from farmers' fields and acquire the ability to form self-perpetuating populations (Gressel, 2005). Then, when these biotypes invade agricultural fields, they are designated as feral weeds (Gressel, 2005; Ellstrand et al., 2010). Thus, weeds can originate in three ways: 1) from crop varieties (endoferality); 2) from wild biotypes; and 3) from hybrids between wild and crop taxa (exoferality) (Baker, 1974; Ellstrand et al., 2010; Huang et al., 2017). As cultivated and wild biotypes show divergence in many traits, weeds of cultivated or wild origin may receive a different set of pre-adapted traits. For example, endoferal weeds may harbor traits that are pre-adapted to agricultural environments (e.g., fast growth, early flowering, or herbicide resistance), but seed dormancy or shattering may be necessary for the formation of self-perpetuating populations (Ellstrand et al., 2010; Vercellino et al., 2018). Weeds originating from wild biotypes may harbor traits pre-adapted to form self-perpetuating populations, but they need to evolve adaptation to agricultural environments (Baker, 1974; Kane and Rieseberg, 2008; Kreiner et al., 2018). Weeds from crop-wild hybrids harbor a combination of traits from both parents, which may facilitate rapid adaptation to agricultural environments (Casquero et al., 2013; Le Corre et al., 2020; Pandolfo et al., 2018; Presotto et al., 2017; Vigueira et al., 2019).

A powerful technique for studying rapid evolution is the “resurrection approach” (Franks et al., 2018). This approach consists in reviving ancestors from stored propagules (often dormant seeds) and comparing them with descendants under common conditions. In this way it is possible to evaluate the phenotypic and genetic changes in response to the most important selective forces (e.g. drought stress, or global warming) that may have occurred during the time between collections (Franks et al., 2018; Kuester et al., 2016; Weis, 2018). This approach has been used to study rapid phenotypic and/or genetic changes in plants in response to drought (Franks et al., 2016), and herbicide use (Kuester et al., 2016), demonstrating that the evolution of adaptive traits may occur in a few generations (<10) if the selective forces are strong enough.

Wild sunflower (*Helianthus annuus* L.) is an excellent model system to study the role of crop-wild hybridization on the evolution of weeds, due to its wide distribution in field margins, the

natural hybridization with cultivated sunflower (Snow et al., 1998; Ureta et al., 2008), the large divergence in ecologically relevant traits between crop and wild (Hernández et al., 2017; Presotto et al., 2011; Wills and Burke, 2007), and the recurrent evolution of weedy biotypes, both in native and introduced areas (Casquero et al., 2013; Kane and Rieseberg, 2008; Mayrose et al., 2011; Muller et al., 2011). The repeated use of herbicides in agricultural fields has resulted in the evolution of biotypes resistant to AHAS herbicides (Al-Khatib *et al.* 1998; White *et al.* 2002; Tranel et al., 2021), and more recently to glyphosate (Singh *et al.* 2020; Tranel et al., 2021).

In Argentina, wild sunflower was introduced from North America at least 70 years ago, probably with breeding purposes, and has subsequently spread over the central region (Cantamutto et al., 2008; Poverene et al., 2008). Argentine biotypes harbor high genetic and phenotypic variation, probably due to multiple introductions and post-introduction admixture (Hernández et al., 2019b). In Argentina, several hybrid zones were formed where wild and cultivated sunflower often grow in sympatry (Ureta et al., 2008), however, to our knowledge, no biotypes have invaded agricultural fields in these zones (Mondon et al., 2018). Despite the frequent occurrence of natural crop-wild hybrids in these areas (Mondon et al., 2018; Ureta et al., 2008), no evidence of permanent crop introgression was found (Mondon et al., 2018), probably due to ecological barriers for crop to wild introgression (Mercer et al., 2007; Presotto et al., 2019).

Here, we studied the origin and genetic diversity of a recent weedy biotype collected at Barrow (BRW), in a region with no previous records of wild sunflower. Then, using a resurrection approach, we tested for rapid evolution of weedy traits in this biotype. The specific aims were to 1) investigate the origin and genetic diversity of BRW using one crop-specific mitochondrial marker (CMS-PET1) and 14 nuclear SSR markers, and 2) test whether BRW shows evidence of rapid evolution of weedy traits: seed dormancy, herbicide resistance, and competitive ability. We hypothesize that BRW is a natural crop-wild hybrid, which facilitated rapid evolution towards higher seed dormancy, herbicide resistance, and higher competitive ability through selection on the standing genetic variation.

2. MATERIALS AND METHODS

2.1. Plant material

We focused our study on a recent weedy biotype of *H. annuus*, found at Barrow (BRW; 38°16′ S, 60°07′ W), near Tres Arroyos (Buenos Aires). This is the first *H. annuus* biotype reported as an agricultural weed infesting sunflower and maize crops in Argentina, in a region with no previous records of wild *H. annuus* (Casquero et al., 2013) (Fig. 1).

The agricultural field where BRW was collected is managed by farmers following a summer crop rotation system, alternating sunflower, glyphosate-resistant soybean, and maize (Casquero et al., 2013). When soybean was planted, no weedy sunflower plants were observed in the field (Casquero et al., 2013), probably due to effective chemical control with glyphosate. On the contrary, when sunflower or maize were planted, up to 48,000 weedy sunflowers per ha^{-1} were found (Casquero et al., 2013). For this reason, we hypothesize that AHAS-resistance has contributed to the adaptation of weedy sunflower in the agricultural environment.

First, to investigate the origin and genetic diversity of this weedy biotype, we compared BRW with wild and cultivated sunflower using a crop-specific marker (CMS-PET1) and 14 nuclear SSR markers. Then, using a resurrection approach, we tested for rapid evolution of weedy traits (seed dormancy, herbicide resistance, and competitive ability) in response to agricultural conditions by sampling BRW 10 years apart (2007 and 2017). To test for evolution due to conditions other than the agricultural ones (e.g., due to global change or genetic drift), we included three ruderal biotypes in the resurrection approach: Colonia Baron (BAR; 34°47′ S, 68°15′ W), Diamante (DIA; 32°03′ S, 60°38′ W) and Río Cuarto (RCU; 33°09′ S, 64°20′ W) (Fig. 1), all three were collected 10 years apart (2007 and 2017).

In both samplings, we followed the recommendations of Franks *et al.* (2018). Seeds from 50-150 plants were collected at the same site using GPS location (Fig. 1). To reduce the so called “invisible fraction bias” (Weis, 2018), consisting in mean phenotypic variation due to non-random mortality during storage, the achenes (hereafter seeds) for the ancestral biotypes (2007) were regenerated once (in 2011) in a common garden through controlled pollination of 25-50 individuals. To avoid selection of seed traits during regeneration and seed production in the common garden, the seeds were stratified on wet paper at 5°C for one week to overcome seed dormancy and then they were planted on trays on sphagnum peat-based substrate. The seedlings were grown in the greenhouse at ~25°C up to the 4-6 leaf stage and then were

transplanted. Seeds for each biotype were produced under controlled pollination of the heads of at least 25 maternal plants, following Hernández et al., (2019a).

Figure 1. Geographic location of ruderal biotypes (in blue) and the agrestal biotype (BRW, in brown) used in this study. Blue dots indicate biotypes not included in this study. Note that most of the biotypes are distributed over the central region and no ruderal biotypes were found close to BRW. A: typical ruderal biotype (BAR) growing on a roadside near to Colonia Baron (BAR, La Pampa province). B: the agrestal biotype growing in a maize field near to Tres Arroyos (BRW, Buenos Aires province). Both photos are from the last collection trip in March 2017, sampling all populations except for LMA and MAG.

2.2. Biotypes sampled for genotyping and population genetic analysis

The discovery of cytoplasmic male sterility (CMS) along with fertility restorer (Rf) genes resulted in the production of hybrid seed on a large scale, which lead to large increases in crop yields due to heterosis. In sunflower, hybrid seed production is based on the CMS-PET1 cytotype, discovered by Leclercq (1969) and a few Rf genes. The CMS-PET1 cytotype is present in the female parents to ensure cross-pollination while Rf genes are carried by male parents in homozygosis to restore pollen fertility in the hybrid progeny. As the cytoplasm is maternally inherited, all cultivars carry the CMS-PET1 cytotype, whereas it is absent in wild sunflowers (Rieseberg et al., 1994). Thus, markers differentiating CMS-PET1 from non-CMS cytotypes can

be used to infer the cultivated or wild origin of weeds (Garayalde et al., 2015; Muller et al., 2011; Rieseberg et al., 1994).

First, we used the CMS-PET1 marker to discern the cultivated or wild origin of BRW. We genotyped 32 samples from Argentina (8 individuals each for BAR, BRW, DIA, and RCU), two commercial cultivars (Cacique CL and HS03) as positive controls, and 10 samples from North American biotypes: three individuals from North Dakota (ND; PI 586888) and Texas (TX; PI 613728), and four individuals from California (CA; PI 413131). To detect the occurrence of the CMS-PET1 cytotype, we used a duplex PCR strategy, *orfC* primers were used to indicate the presence/absence of the CMS-PET1 cytotype, and *coxIII* was included as a positive control (Garayalde et al., 2015; Rieseberg et al., 1994). Primer sequences, PCR conditions, and the expected size of PCR products for *orfC* and *coxIII* can be found in Garayalde *et al*. (2015).

To study the genetic diversity and population structure, we collected DNA from 79 individuals, 60 belonging to six wild biotypes collected in central Argentina (hereafter ruderal biotypes; Fig. 1) used in Hernández et al., (2019b), plus 10 individuals from BRW, and nine cultivated inbred lines from different origins (Table S1). BAR, BRW, DIA, and RCU biotypes were collected in 2017, AAL in 2010, and LMA and MAG in 2007, by group members following the same collection protocols. Individuals were genotyped using 14 nuclear microsatellite markers previously used by Mandel et al., (2011), that were selected based on neutrality and the genetic map position (one marker in 14 out 17 sunflower linkage groups). A detailed description of the DNA extraction and SSR genotyping protocols can be found in Hernández et al., (2019b). Three individuals (one from BRW and two from RCU) with missing data at more than three loci were removed from further analyses.

The effective number of alleles per biotype was calculated with rarefaction using the *hierfstat* R package (Goudet, 2005); the Shannon´s information index and unbiased gene diversity were calculated at the biotype level using GenAlEx v. 6.5 (Table 1; Peakall and Smouse 2012). Population structure was assessed using analysis of molecular variance (AMOVA), as implemented in GenAlEx, to hierarchically partition the genetic variation and estimate Wright's F_{ST}.

Bayesian clustering, using the STRUCTURE v.2.3.4 software (Pritchard et al., 2000) was used to assign multi-locus genotypes into clusters. We implemented the admixture model with correlated allele frequencies and the remaining parameters set as default over 10 independent runs for each *K* (1-8) with 100,000 MCMC iterations and a burn-in period of 50,000 iterations.

The number of clusters (*K*) was chosen based on the method of Evanno *et al*. 2005. Replicate STRUCTURE runs were combined and visualized with the interactive version of the *pophelper* R package (Francis, 2017). The population structure was also studied using discriminant analysis of principal components (DAPC), with the *adegenet* R package (Jombart et al., 2010).

2.3. Germination experiments

For the gemination experiments, we used four biotypes sampled 10 years apart (2007 and 2017): BAR, BRW, DIA, and RCU. Two germination experiments were conducted in two different years. For experiment 1 (2016/17), seeds of the ancestral biotypes (2007) were produced in a common garden at the Agronomy Department, Universidad Nacional del Sur, Bahia Blanca, Argentina (38°41´38´´S, 62°14´53´´W), whereas descendant biotypes (2017), 50-100 plants per biotype, were collected from their local environments in a three-day collection trip (Fig. 1). For experiment 2 (2017/18) all the seeds were produced in a common garden at the Agronomy Department, Universidad Nacional del Sur, to control for maternal environmental effects.

The germination experiments were carried out following Hernández *et al*. (2019a). Briefly, germination was evaluated at two different times: two months (T1) and eight months (T2) after harvest, and at three constant temperatures (10 °C, 20 °C, and 30 °C). After harvest, the seeds used in T1 were stored in tri-laminar aluminum bags to protect the seeds from humidity, at room temperature (25 °C) for 2 months. Then, the seeds used in T2 were placed in tri-laminar aluminum bags in a growth chamber at 5 °C for six months. Twenty-five seeds per replicate and biotype were placed on filter paper in petri dishes and moistened with distilled water, with a 12 h photoperiod and they were counted periodically at 2–3 days intervals for 16 days. Replicates were arranged in four complete blocks (a rack of the growth chamber). For each experiment, the germination percentage (GRP) was calculated following Hernández et al., (2019a).

Data were analyzed with generalized linear models using PROC GLM in SAS (SAS University edition; SAS Institute Inc., Cary, NC). Each experiment and time were analyzed separately resulting in four analyses. The single effects were the collection Year (2007 and 2017), Biotype (BAR, BRW, DIA, and RCU), and Temperature (10, 20, and 30°C), besides the two-way interactions: Year*Biotype, Year*Temperature, Biotype*Temperature, and the three-way interaction Year*Biotype*Temperature. Due to the high homogeneity in the blocks (racks of the growth chamber) we did not include the block effect in the analyses. All effects were considered as fixed. When the Year*Biotype interaction was significant, the least square means of each

biotype were compared between years, and when the Year*Biotype*Temperature interaction was significant, the least square means of each biotype and temperature were compared between years. Due to the lack of seeds, some biotypes were not evaluated at all three temperatures in all the experiment and time combinations.

Meta-analysis was used to test whether an overall phenotypic change in GRP exists in different biotypes using OpenMEE software (Wallace et al., 2017). First, we calculated the standardized mean difference (Hedge´s d) between the biotypes collected in 2017 and 2007 for each biotype, temperature, experiment, and time as the difference between GRP of biotypes collected in 2017 and 2007 divided by its pooled and weighted standard deviation (Wallace et al., 2017). Then, we performed separate analyses for each experiment. Random-effects models were used to combine the effect sizes of different treatments (three temperatures and two times), as well as their 95 % confidence intervals. Positive Hedge´s d values indicate an overall higher GRP (lower dormancy) of biotypes collected in 2017 whereas negative values indicate the contrary. Differences between collection years per biotype were considered significant when the 95 % confidence interval did not overlap zero.

2.4. Competition experiment

A target-neighborhood design was used to evaluate the competitive responses of three sunflower biotypes at three levels of maize competition in a factorial experimental design with five replicates. Competition levels consisted in 0, 1, and 3 maize plants per pot (Treatments 1, 2, and 3, respectively), and biotypes were BAR (collected in 2017), and BRW sampled in 2007 (hereafter BRW_{07}) and 2017 (hereafter BRW_{17}). For Treatments 2 and 3, the maize plants were established by sowing one and three seeds, respectively, in 15 cm diameter plastic pots (2.5 L) while no maize seeds were sown for the Treatment 1. At the two-leaf stage of maize, sunflower plants were established by sowing one pre-germinated sunflower seed per pot. The experiment was carried out in a greenhouse (25 °C ± 3 °C), with light provided by six 36 W fluorescent tubes located 50 cm above plants, and the pots were watered manually to keep them at field capacity throughout the experiment. Three variables (plant height, leaf width and leaf length) were measured four times on sunflower plants on 13, 17, 22, and 30 days after sowing, and the leaf size was estimated as leaf width*leaf length for each time. At the end of the experiment (30 days after sowing), the aboveground biomass of sunflower plants was collected by cutting the entire plants at ground level, drying the tissue at 60 °C for 7 days, and weighing it.

Data were analyzed with generalized linear models using PROC GLM in SAS. Each time was analyzed separately resulting in four analyses per variable (plant height, leaf length, leaf width, and leaf size) and one analysis for aboveground biomass (only measured at the end of the experiment). For each variable, the single effects were competition Treatment (three levels), Biotype (BAR, BRW_{07}, and BRW_{17}), and the Treatment*Biotype interaction. All effects were considered as fixed. When the Treatment*Biotype interaction was significant, the least square means of the biotypes were compared within each treatment. All pairwise comparisons were performed with Bonferroni-adjusted P values.

2.5. Herbicide resistance experiment

To evaluate herbicide resistance in BRW, the response of BRW_{07} and BRW_{17} to imazapyr was evaluated. The progeny of a herbicide resistant cultivar (Cacique CL, hereafter IMI) and a known susceptible biotype (BAR) were included as resistant and susceptible controls, respectively. For the screening, plants were established by sowing 51 (BAR and IMI) or 135 (BRW_{07} and BRW_{07}) pre-germinated seeds in 200-cell plastic trays (50 cc per cell) in a complete randomized design with three replicates of 17 (BAR and IMI) or 45 (BRW_{07} and BRW_{07}) plants, totaling 372 plants. At the two-leaf stage, Imazapyr was applied at 1.2 X the recommended rate (X = 210 mL ha^{-1}) and the survival rate was recorded 18 and 35 days after herbicide application. Plants were grown in a greenhouse at 20-25 °C, from sowing to the end of the experiment.

3. RESULTS

3.1. Origin and genetic diversity of the weedy biotype

Cytoplasmic analysis shows that CMS-PET1 cytotype was present in all the individuals of BRW (n = 8), but it was absent in all wild individuals from either Argentina (n = 29) and United States (n = 10), indicating a feral origin of BRW.

To find out whether the origin of BRW is endoferal (from a crop escape) or exoferal (resulting from crop-wild hybridization), we analyzed the genetic diversity and population structure of BRW as well as the wild biotypes and cultivated lines (CROP), using 14 nuclear SSR markers. The effective number of alleles varied from 2.38 in CROP to 3.07 in RCU (Table 1). Similarly, Shannon´s information index varied from 0.82 in CROP to 1.18 in RCU. The unbiased expected heterozygosity (uHe) varied from 0.50 in CROP and DIA to more than 0.60 in BAR, BRW, and RCU (Table 1). The number of private alleles (NPA) varied from 2 in DIA to 13 in AAL, being intermediate in BRW (NPA=6; Table 1). When BRW was excluded from the analysis, the

number of private alleles (NPA^2) varied from 5 in CROP and LMA to 15 in AAL (Table 1), and the NPA^2 found in BRW (Int. PA) was 0 or 1 for most biotypes, except for DIA (4 alleles from 4 loci) and BAR (3 alleles from 2 loci) (Table 1). BRW exhibited at least 20% more genetic diversity than CROP, and similar values to RCU and BAR for any genetic diversity measure (Table 1) suggesting that wild hybridization increased the genetic diversity of the feral BRW.

Table 1. Measures of genetic diversity per biotype.

Biotype	N	Ne	I	uHe	NPA	NPA^2	Int. NPA	F_{ST}
BRW	9	3.00	1.13	0.62	6			
RCU	8	3.07	1.18	0.65	10	10	0	0.119
BAR	10	3.01	1.14	0.61	8	11	3	0.102
AAL	10	2.85	1.07	0.55	13	15	2	0.121
MAG	10	2.60	0.96	0.55	9	9	0	0.174
LMA	10	2.56	0.92	0.52	4	5	1	0.137
DIA	10	2.50	0.86	0.50	2	6	4	0.163
CROP	9	2.38	0.82	0.50	4	5	1	0.074

N: number of genotyped plants. Ne: effective number of alleles (calculated with rarefaction); I: Shannon´s information index; uHe: unbiased expected heterozygosity; NPA: number of private alleles; NPA^2: number of private alleles excluding BRW from the analysis; Int. NPA: number of private alleles found in BRW; F_{ST}: pairwise F_{ST} with BRW.

The population structure and the genetic relationship between the biotypes were evaluated using three independent approaches (pairwise F_{ST}, STRUCTURE, and DAPC). The pairwise F_{ST} between BRW and the other biotypes ranged from 0.07 (BRW-CROP) to 0.17 (BRW-MAG) (Table 1). The lowest F_{ST} values were observed with CROP and BAR, whereas the highest were with DIA and MAG (Table 1). STRUCTURE analysis suggests three, followed by five genetic groups (Fig. S1A, 2A). At K=3, individuals of BRW grouped with CROP and DIA individuals (Fig. 2A). At K=5, individuals of BRW showed evidence of an admixture between

BAR and CROP (Fig. 2A). DAPC assigned individuals into four groups (Fig. S1B, 2B). Group 1 includes individuals from MAG, BAR, and RCU, while group 2 includes all the individuals from BRW and most (eight out of nine) cultivated lines and BAR individuals (seven out of ten) (Fig. 2C). Moreover, group 3 includes most individuals of AAL and LMA as well as two individuals of RCU, whereas group 4 includes all the individuals from DIA and one CROP line (PMI3) (Fig. 2C). All of this suggests that BRW is a natural crop-wild hybrid between CROP and BAR parents.

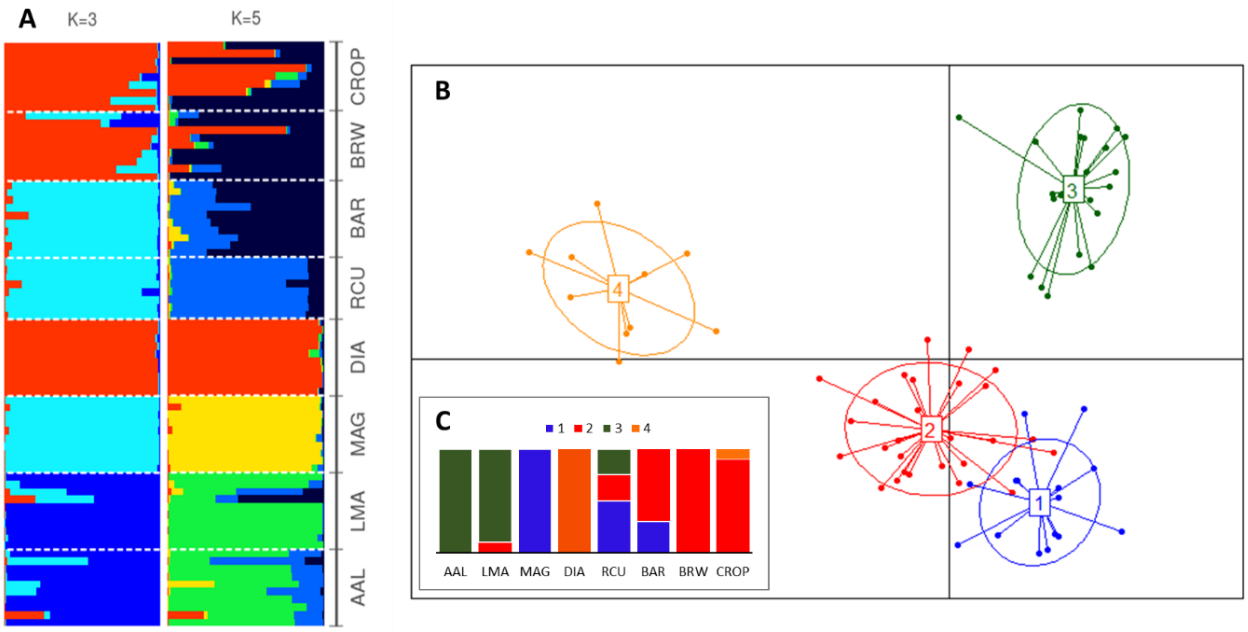

Figure 2. Population structure of wild, cultivated, and weedy sunflower. A: STRUCTURE output; B: DAPC output; C; proportion of individuals assigned to each DAPC group. Wild biotypes: AAL, LMA, MAG, DIA, RCU, and BAR; weedy: BRW; cultivated: CROP.

3.2. Germination experiments

To test for phenotypic evolution between the years of collection, we explored the Year*Biotype and Year*Biotype*Temperature interactions.

We observed significant Year*Biotype*Temperature interactions in all four experiment and time combinations ($P = 0.06$ for Experiment 1 and Time1, $P < 0.01$ for the rest), indicating that some biotypes differ between collection years, and these responses varied with temperature. In Experiment 1, BRW_{17} showed a significantly lower GRP than BRW_{07} in all six experimental

combinations (Table 2), whereas DIA and RCU collected in 2017 had a lower GRP than those collected in 2007, but only at 20 °C (Table 2). In addition, at Time 1 and 10°C, DIA_{17} showed a higher GRP than DIA_{07} (Table 2). Similar results were observed in experiment 2, when all the seeds were produced in a common garden. BRW_{17} showed a significantly lower GRP than BRW_{07} in all four combinations with some germination, whereas DIA_{17} and RCU_{17} showed a lower GRP than DIA_{07} and RCU_{07} at 20°C (Table 2). We also observed significantly lower GRP in DIA_{17} than in DIA_{07} at 10°C in both years (Table 2).

Table 2. Germination percentage (GRP) for each biotype measured in two different years in two experiments at three temperatures.

Biotype	Experiment	Time	Temperatures					
			10 °C		20 °C		30 °C	
			2007	2017	2007	2017	2007	2017
BAR	1	1	-	-	0.10 ± 0.1	0.17 ± 0.1	-	-
BRW			**0.54 ± 0.2**	**0.34 ± 0.1**	**0.90 ± 0.0**	**0.61 ± 0.1**	**0.66 ± 0.1**	**0.11 ± 0.1**
DIA			**0.07 ± 0.1**	**0.23 ± 0.1**	**0.92 ± 0.1**	**0.76 ± 0.1**	0.30 ± 0.2	0.29 ± 0.1
RCU			0.20 ± 0.1	0.29 ± 0.2	0.42 ± 0.0	0.45 ± 0.1	0.04 ± 0.0	0.04 ± 0.0
BAR		2	-	-	**0.19 ± 0.1**	**0.43 ± 0.0**	-	-
BRW			**0.85 ± 0.1**	**0.67 ± 0.1**	**0.86 ± 0.1**	**0.67 ± 0.1**	**0.61 ± 0.1**	**0.29 ± 0.0**
DIA			0.36 ± 0.2	0.39 ± 0.1	**0.88 ± 0.1**	**0.63 ± 0.0**	0.15 ± 0.1	0.24 ± 0.1
RCU			0.51 ± 0.2	0.48 ± 0.1	**0.87 ± 0.1**	**0.58 ± 0.1**	0.13 ± 0.1	0.07 ± 0.1
BAR	2	1	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0
BRW			0.02 ± 0.0	0.02 ± 0.0	**0.77 ± 0.1**	**0.02 ± 0.0**	-	-
DIA			**0.21 ± 0.1**	**0.04 ± 0.0**	**0.91 ± 0.1**	**0.60 ± 0.2**	0.01 ± 0.0	0.05 ± 0.0
RCU			0.01 ± 0.0	0.01 ± 0.0	**0.53 ± 0.1**	**0.28 ± 0.1**	0.01 ± 0.0	0.01 ± 0.0
BAR		2	0.01 ± 0.0	0.07 ± 0.1	0.02 ± 0.0	0.06 ± 0.0	0.01 ± 0.0	0.01 ± 0.0
BRW			**0.93 ± 0.0**	**0.48 ± 0.2**	**0.99 ± 0.0**	**0.43 ± 0.1**	**0.33 ± 0.1**	**0.02 ± 0.0**
DIA			**0.70 ± 0.2**	**0.13 ± 0.1**	**0.97 ± 0.0**	**0.63 ± 0.2**	0.10 ± 0.1	0.07 ± 0.1
RCU			0.13 ± 0.1	0.07 ± 0.1	**0.78 ± 0.1**	**0.48 ± 0.1**	0.15 ± 0.2	0.04 ± 0.1

Experiment 1: seeds produced in a common garden (2007) and local environments (2017) during the 2016/17 growing season; Experiment 2: all seeds (2007 and 2017) produced in a common garden during the 2017/18 growing season. Time 1: two months after harvest; Time 2: eight months after harvest, with six months stored at 5°C. Significant differences ($P < 0.05$, adjusted for multiple comparisons) between collection years for each experiment, time, and temperature, in bold.

Because the Year*Biotype*Temperature interaction for GRP was significant in all four experiment and time combinations, we used meta-analysis tools to find out whether an overall phenotypic change (per temperatures) existed between the collection years. We found support for an overall and significant decrease of GRP with the collection year in BRW ($P < 0.0001$ and $P = 0.005$ for experiments 1 and 2, respectively), but no significant effects were found in BAR, DIA, or RCU (Fig. 3A). Despite not being significant, in experiment 2 (Fig. 3B), DIA and RCU showed an overall decrease in GRP ($P = 0.051$ and $P = 0.052$, for DIA and RCU, respectively).

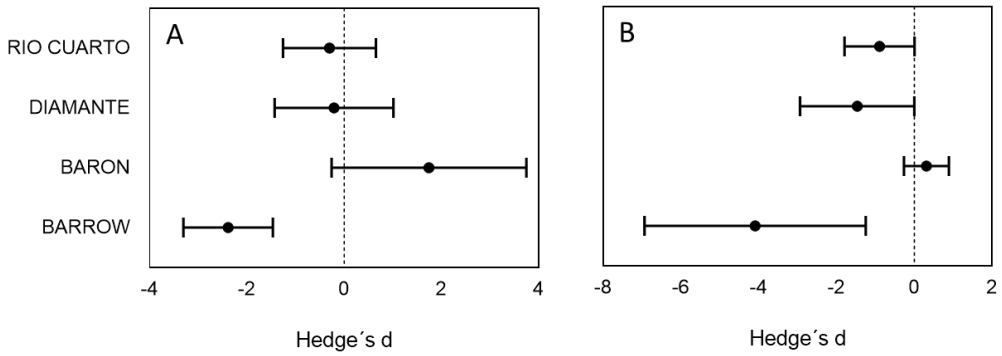

Figure 3. Effect of the collection year (2007 vs. 2017) on the germination percentage (GRP) per temperature and time in experiments 1 (A) and 2 (B). Positive Hedge´s d values indicate an overall higher GRP of biotypes collected in 2017 while negative values indicate the contrary. Effects were considered as significant when 95% confidence interval did not overlap zero.

3.3. Interspecific competitive ability

We observed a high and positive correlation between leaf traits measured at different times and the aboveground biomass (Table S2). Plant height showed high correlations between times, but weak correlations with aboveground biomass and leaf traits (Table S2). To avoid redundancy, we retained three variables (plant height, leaf size, and aboveground biomass) measured at the end of the experiment (Time 4).

For plant height, we observed significant differences between the biotypes, but no significant Treatment effect or Biotype*Treatment interaction (Fig. 4A). For leaf size and biomass, we observed significant Biotype and Treatment effects, and Biotype*Treatment interactions (Fig. 4B-5C). Without any competition from maize (Treatment 1), both BRW_{07} and BRW_{17} showed higher leaf size and biomass than BAR (Fig. 4B-4C), with some differences between BRW_{07} and BRW_{17}, especially for leaf size (Fig. 4B). Under competition with one maize plant (Treatment 2),

the differences between BRW_{07} and BRW_{17} disappeared whereas differences with BAR were smaller (Fig. 4B-4C). In treatment 3, all three biotypes showed similar values (Fig. 4B-4C).

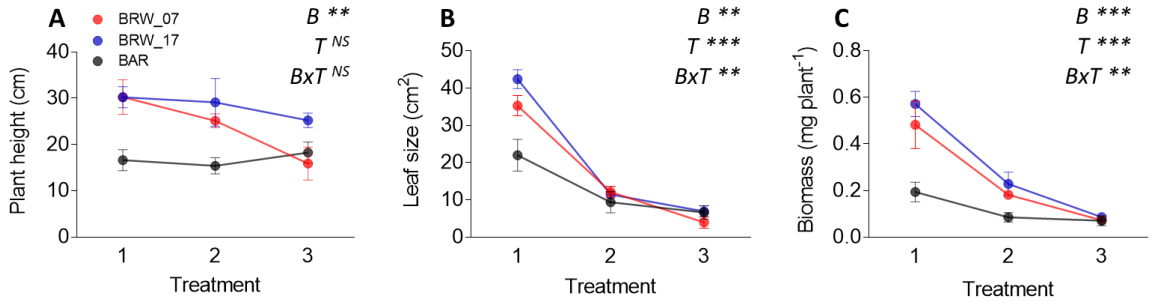

Figure 4. Phenotypic variation of three sunflower biotypes in response to competition. BAR: Colonia Baron collected in 2017; BRW_{07}: Barrow collected in 2007; BRW_{17}: Barrow collected in 2017. Competition treatments consist of zero, one, and three maize plants per pot (Treatments 1, 2, and 3, respectively). One plant of sunflower was sown per pot when maize plants were at the two-leaf stage.

3.4. Herbicide resistance

At a 1.2 X the recommended rate of imazapyr, plant survival was nil for BAR (0/46) and BRW_{07} (0/126) and very low for BRW_{17} (1/129), whereas it was high for IMI (41/48).

4. DISCUSSION

4.1. On the origin of BRW

Understanding the origin and evolution of agricultural weeds is critical for designing better weed management strategies, especially for preventing the emergence of novel biotypes and the escape of genes/alleles from cultivated species. Our results, based on the CMS-PET1 confirmed the feral origin of BRW proposed by Casquero *et al.* (2013). All individuals of BRW harbor the CMS-PET1 cytotype, used in commercial hybrid seed production (Muller et al., 2011; Rieseberg et al., 1994). The CMS cytotype has previously been used to confirm the feral origin of weedy sunflower biotypes (Muller et al., 2011) in Europe. In addition, we found that BRW shows greater genetic diversity than CROP, with a diversity as high as the wild biotypes, and it harbors many alleles that are not present in our cultivated sample. All of this supports a crop-wild origin for BRW (Muller et al., 2011). Although our sampling of the cultivated lines was somewhat limited, indices for genetic diversity are representative of those observed in a worldwide collection of sunflowers (Mandel et al., 2011). For example, for cultivated lines, we

observed Ne=2.38 and uHe=0.5 (Table 1) and Mandel et al., (2011) reported Ne=2.65 and uHe=0.43.

The weedy BRW was collected from a region with no previous records of wild sunflower (Fig. 1; Cantamutto et al., 2008; Poverene et al., 2008), thus, where did hybridization take place? Hybrid zones, where crop and wild relatives grow in sympatry are potential sources of crop-wild hybrids. Two of our sampled biotypes often grow in sympatry with the crop: AAL in Adolfo Alsina, and BAR in Colonia Baron, and natural crop-wild hybrids are often observed there (Mondon et al., 2018; Ureta et al., 2008). In Argentina, the genetic diversity of wild sunflower is geographically structured (Hernández et al., 2019b), making population genetic approaches useful for identifying the geographic source of BRW. Of the two hybrid zones, BAR is the most probable source. All the approaches we used (pairwise F_{ST}, private alleles, and clustering analyses) showed that BAR was the wild biotype that was genetically closest to BRW. Surprisingly, we found some genetic similarity between DIA and BRW, especially for private alleles and from the STRUCTURE clustering. Although the origin of DIA is unclear (Hernández et al., 2019b), this biotype does not grow in sympatry with cultivated sunflower (Poverene et al., 2008). A probable origin of DIA is the intentional introduction by European migrants during early 1900´s (Hernández et al., 2019b), therefore, it is possible that both BRW and DIA have a feral origin: BRW from modern cultivars (all plants harbor the CMS-PET1 cytotype), and DIA from landraces or open pollinated varieties (no plants harbor the CMS-PET1 cytotype). On the other hand, the farmer who manages the field where BRW was collected indicated that the first plants of multiheaded sunflower were seen after renting a harvest machine from an area close to Colonia Baron, where BAR is naturalized. As no wild plants were observed in BRW but crop-wild hybrids are often observed in BAR (Mondon et al., 2018; Ureta et al., 2008), we hypothesize that hybridization occurred in Colonia Baron, and then hybrid seeds were accidentally introduced into Barrow.

Feral weeds have been reported in a dozen cultivated species (reviewed in Ellstrand *et al.* 2010). In sunflower, feral forms have been found in agricultural fields across southern Europe, where the wild species is absent (Muller et al., 2011). There are three similar features between BRW and European biotypes (Casquero et al., 2013; Muller et al., 2011; Presotto et al., 2017): 1) the exoferal origin, in both cases natural crop-wild hybrids were accidentally introduced to farmers' fields; 2) allopatry with wild sunflower, gene flow with wild sunflower was disrupted when crop-wild hybrids were introduced to a novel region; and 3) the adaptation to agricultural environments, an ecological transition for a typically ruderal species (Kane and Rieseberg,

2008; Poverene et al., 2008; Snow et al., 1998). While weedy biotypes have also evolved from wild ruderals (Kane and Rieseberg, 2008; Mayrose et al., 2011), crop-wild hybrids are rarely established in ruderal environments, even in hybrid zones (Mondon et al., 2018; Poverene et al., 2008). This indicates that crop-wild hybridization is not required but it may facilitate the ecological transition from ruderal to agricultural habitats. Recently, we showed that wild-like traits are under selection in both ruderal and agricultural habitats, but selection is generally weaker in the latter (Presotto et al., 2019). A weaker selection for wild-like traits can explain why crop-wild hybrids are (apparently) more successful away from hybrid zones. While the combination of specific traits from wild and cultivated parents may explain the success of crop-wild hybrids in agricultural environments (Mercer et al., 2007; Presotto et al., 2017), hybridization followed by transgressive segregation can facilitate major ecological transitions (Rieseberg et al., 2003). Further studies using natural and synthetic hybrids are needed to directly test the role of hybridization on evolution under agricultural conditions.

4.2. Standing genetic variation allowed rapid evolution of seed dormancy

We found strong support for rapid evolution of seed dormancy in BRW, but not for herbicide resistance or competitive ability. For seed dormancy, in both germination experiments, and per temperatures, BRW_{17} showed much higher dormancy than BRW_{07}. Similarly, seed dormancy that has been observed in feral forms of rice (Huang et al., 2017), and radish (Vercellino et al., 2019) is probably the result of rapid evolution during de-domestication. Due to its crucial ecological role and its early expression in the life cycle, seed dormancy is expected to be under especially strong selection (Huang et al., 2010). Therefore, we recently reconstructed the genetic background of BRW by crossing a CROP as the female with BAR as the male and we observed an overall low seed dormancy with cascading effects leading to high autumn emergence, low overwinter survival, and poor establishment in the field (Hernández et al., 2021). This indicates that if genetic variation is present, selection against low dormant phenotypes can be strong.

Seed dormancy may evolve from the standing genetic variation or new mutations, however, evolution from the standing genetic variation is likely to be much faster than from new mutations, especially when multiple small-effect genes are involved (Kreiner et al., 2018). In sunflower, no major genes of large effect have been identified for seed dormancy but many small-effect QTLs (Brunick, 2007; Gandhi et al., 2005), making the evolution of seed dormancy through new mutations in multiple regions very unlikely. Hybridization between wild and cultivated sunflowers leads to high phenotypic and genetic variation on which selection can act

(Muller et al., 2011; Presotto et al., 2019). After hybridization, wild and cultivated alleles starting at high frequencies and novel recombinations may produce transgresive phenotypes (Rieseberg et al., 2003). In a recent seed bank experiment, we observed that most BRW_{07} seeds germinated in the first year (up to 80%), which is consistent with the low dormant phenotype observed here, but also a small fraction of seeds remained dormant and viable in the soil for at least four years (Presotto et al., 2020), indicating that BRW_{07} had substantial genetic variation for seed dormancy on which selection could act. In summary, rapid evolution of seed dormancy in BRW was probably facilitated by strong directional selection on the standing genetic variation.

On the other hand, we also found support for rapid evolution of dormancy in ruderals; the biotypes from DIA and RCU collected in 2017 showed significantly higher dormancy at 20°C than the biotypes collected in 2007. To our knowledge, these biotypes did not experience any major habitat changes since their introduction at least 70 years ago (Cantamutto et al., 2008; Poverene et al., 2008). So, what forces are driving rapid evolution in the ruderals? As these biotypes are distributed in patches at least 100 km apart from each other, and harbor thousands of individuals (Cantamutto et al., 2008; Poverene et al., 2008), it is unlikely that genetic drift and gene flow have played a role in the evolution of seed dormancy. From our data, it is not possible to discern whether the evolution of dormancy over the last decade is part of a continous process of local adaptation that began with the introduction of sunflower into Argentina (Hernández et al., 2019a), or whether it occured in response to recent climatic changes after biotypes became locally adapted. Unfortunately we do not have any viable seeds of these biotypes older than 15 years in order to directly evaluate phenotypic changes over a wider time frame. However, alternative approaches like herbarium genomics could be used for tracking changes in genome-wide and functional genetic diversity since the introduction of wild sunflower in Argentina.

4.3. The evolution of herbicide resistance is limited due to lack of standing genetic variation

The repeated use of AHAS-inhibiting herbicides in agricultural fields has resulted in the evolution of AHAS-resistant biotypes in many species, including weedy sunflower (Al-Khatib *et al.* 1998; White *et al.* 2002; Tranel et al., 2021). Here, we found no support for evolution of herbicide resistance, as only one individual (<1 %) of BRW_{17} survived the 1.2 X rate of imazapyr and no individuals of BRW_{07} survived. At this rate, both homozygous and heterozygous plants carrying the resistant allele should survive (Presotto et al., 2012), indicating that the resistant allele is at a very low frequency in BRW_{17}. AHAS-resistance is partially dominant, so cultivars with the Clearfield® technology are homozygous for the resistant allele, implying that this allele starts at a high frequency in crop-wild hybrids when the cultivated parent is AHAS-resistant. In

addition, no fitness costs have been reported in sunflower (Vrbnicanin et al., 2017). All of this (high initital frequency, dominance, and no fitness costs) makes the rapid loss of the resistant allele due to drift or negative selection very unlikely, and implies that wild and cultivated parents of BRW were susceptible to AHAS herbicides.

The lack of standing genetic variation for herbicide resistance probably limited its evolution which shows that BRW has developed other mechanisms to adapt to agricultural environments. Under experimental field conditions, we observed that commercial rates of imazapyr adequately control ruderal and weedy sunflower at the 2-6 leaf stages, but at later developmental stages the herbicide only kills the main bud and most plants survive by developing the axillary buds, which partially restores fitness (unpublished data). This kind of tolerance is probably facilitated by the rapid emergence of wild and weedy sunflower after soil disturbance (Presotto et al., 2020) and the rapid initial growth of BRW (Casquero and Cantamutto, 2016; Presotto et al., 2017). Other mechanisms, such as late emergence, that avoid herbicide application (Darmency et al., 2017) cannot be ruled out, however, residual effects of the AHAS herbicides (Tranel and Wright, 2002) along with very low emergence of sunflower from the seed bank after spring (Presotto et al., 2020) make this mechanism less probable.

For competitve ability, we found no differences between BRW_{07} and BRW_{17}, indicating that this trait has not changed over the last decade. However, both BRW_{07} and BRW_{17} grew faster than BAR, suggesting that traits related to rapid growth were already present in the biotypes collected in 2007. Cultivated and wild sunflower largely differ in early growth (Hernández et al., 2020; Kost et al., 2015; Mercer et al., 2007), and phenotypic selection analyses suggest that an early rapid growth is advantageous in agricultural environments, therefore, it is expected to be selected rapidly (Kost et al., 2015; Presotto et al., 2019). In previous studies using BRW_{07}, we showed that early rapid growth has probably evolved at the expense of lower stress tolerance in this biotype (Casquero and Cantamutto, 2016; Presotto et al., 2017), a similar pattern to that observed in North American weedy biotypes (Mayrose et al., 2011). Although we found no differences under competition with maize, the treatments we chose may represent an extreme competition, and also a low-resource environment, more typically found in non-agricultural than in agricultural environments (Mercer et al., 2007; Presotto et al., 2017).

The resurrection approach is a one of the most powerful techniques for directly testing for rapid evolution (Franks et al., 2018; Weis, 2018). In our study, we found strong support for rapid evolution towards higher seed dormancy in BRW. Although this approach does not distinguish selection from genetic drift or gene flow (Franks et al., 2018), we gathered evidence in favor of

selection. Firstly, the phenotype changed in the predicted sense, towards higher seed dormancy (Darmency et al., 2017; Hernández et al., 2021; Presotto et al., 2020). Secondly, the census size of both ancestor and descendant biotypes (>10,000 individuals) and the effective population size of the descendant biotype (inferred through genetic markers) are very large, minimizing the influence of genetic drift on phenotypic evolution (Kreiner et al., 2018). Thirdly, although gene flow between weedy and cultivated sunflower probably occurred in the time frame between the collections, the cultivated sunflower showed mostly no, or low, dormancy (Hernández et al., 2017, 2021) and the weedy traits changed in the opposite direction, i.e. dormancy evolved despite, rather than aided by, gene flow. Further studies combining the resurrection approach and population genomics can be used to explore genome-wide changes in allele frequency, scan the genome for signatures of recent selection, and to explore the genetic basis of seed dormancy in weedy sunflower.

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SUPPLEMENTARY MATERIAL

Table S1. Cultivated inbred lines used in the present study.

Line	Origin	Country
R432	INTA	Argentina
QHP1	INRA	France
XA	INRA	France
PMI3	INRA	France
Y7Q	INRA	France
RHA274	USDA	United States
RHA419	USDA	United States
HA335	USDA	United States
PM17	USDA	United States

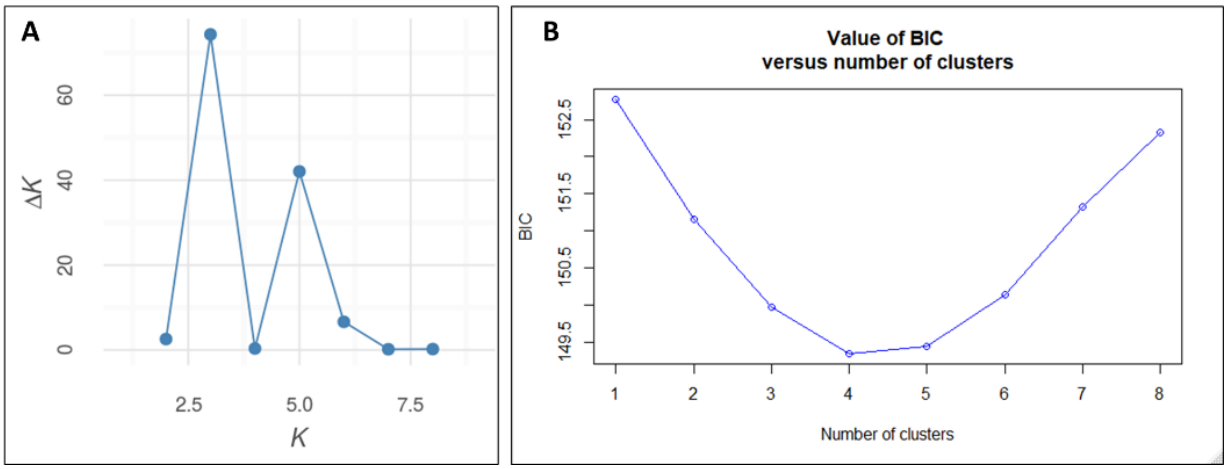

Fig. S1. Best supported number of clusters of STRUCTURE (A) and DAPC (B) analyses.

Table S2. Pearson correlation coefficients (below diagonal) between four traits measured at four times and aboveground biomass at time 4. Above diagonal, P values adjust with Bonferroni method: *** P < 0.0001; **P < 0.01; *P < 0.05; NS P > 0.05. Traits in bold were retained for further analysis.

Variable	1	2	3	4	5	6	7	8	9	10	11	12	**13**	14	15	**16**	**17**
1		**	*	**	***	**	**	**	***	NS	NS	NS	***	NS	*	*	**
2	0.53		***	***	**	***	***	***	**	***	***	***	***	***	***	***	***
3	0.36	0.74		***	**	***	***	***	**	**	***	***	**	*	***	***	***
4	0.41	0.89	0.93		**	***	***	***	**	***	***	***	**	**	***	***	***
5	0.91	0.50	0.42	0.42		**	**	**	***	NS	NS	NS	***	NS	NS	NS	**
6	0.44	0.93	0.68	0.83	0.41		***	***	**	***	***	***	***	***	***	***	***
7	0.47	0.77	0.78	0.80	0.50	0.76		***	**	***	***	***	***	***	***	***	***
8	0.47	0.89	0.78	0.89	0.47	0.91	0.93		**	***	0	***	***	***	***	***	***
9	0.82	0.56	0.49	0.51	0.95	0.48	0.56	0.54		NS	*	NS	0	NS	*	NS	**
10	0.29	0.81	0.56	0.72	0.20	0.90	0.65	0.81	0.27		***	***	**	***	***	***	***
11	0.29	0.82	0.76	0.84	0.25	0.83	0.88	0.92	0.34	0.85		***	**	***	***	***	***
12	0.26	0.84	0.69	0.84	0.20	0.87	0.77	0.89	0.28	0.93	0.96		**	***	***	***	***
13	0.76	0.63	0.51	0.56	0.86	0.60	0.65	0.66	0.94	0.45	0.50	0.45		*	**	**	***
14	0.25	0.64	0.37	0.53	0.12	0.79	0.52	0.68	0.17	0.92	0.74	0.82	0.38		***	***	***
15	0.34	0.77	0.69	0.78	0.27	0.80	0.81	0.87	0.33	0.86	0.95	0.95	0.51	0.82		***	***
16	0.32	0.76	0.60	0.73	0.23	0.81	0.72	0.83	0.26	0.91	0.91	0.95	0.45	0.89	0.98		***
17	0.46	0.84	0.66	0.82	0.41	0.84	0.81	0.91	0.47	0.83	0.89	0.91	0.63	0.74	0.90	0.89	

	Variables	Time
1	Plant height	1
2	Leaf length	
3	Leaf width	
4	Leaf size	
5	Plant height	2
6	Leaf length	
7	Leaf width	
8	Leaf size	
9	Plant height	3
10	Leaf length	
11	Leaf width	
12	Leaf size	
13	**Plant height**	4
14	Leaf length	
15	Leaf width	
16	**Leaf size**	
17	**Aboveground biomass**	