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WILD *Helianthus* species AND WILD-SUNFLOWER HYBRIDIZATION IN ARGENTINA

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SUMMARY

Two wild *Helianthus* species native to North America have been naturalized in Argentina, *H. annuus* ssp. *annuus* and *H. petiolaris*. They grow as adventitious overlapping about 50% of the crop area. Hybridization and introgression between these wild species and sunflower have important biological and practical consequences, the former including homoploid hybrid species formation, and the latter including a possibility of transgene spreading from genetically modified (GM) sunflower cultivars to wild or weedy populations. Wild populations were screened for isozyme and morphological variation. Intermediate plants were found in several locations and subjected to progeny tests. Variability among progeny of each one was compared with the variability in wild accessions of both species. Segregation for phenotypic traits, intermediate phenology and low fertility levels were found in most progenies, accounting for the hybrid origin of their maternal plants. Attempts to quantify gene flow included screening of progenies from *H. petiolaris* populations growing near sunflower crops. Hybrid plants were recognized by morphological traits and reduced fertility. Overall hybridization was about 1%. Hybrid progeny on wild *H. annuus* plants were identified by a crop isozyme marker. A mean frequency of 7% hybridization was found. These results confirm that gene flow occurs among crop and wild *Helianthus* species, and it concerns crop management and environmental impact if release of GM sunflower cultivars is to be authorized in Argentina.

Key words: hybridization, isozymes, morphology, wild sunflower

INTRODUCTION

Two annual species of the genus *Helianthus* (Asteraceae) first described as adventitious in Argentina, have become naturalized in the past 50 years. *Helianthus*

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annuus L. and *H. petiolaris* Nutt. were presumably introduced from their center of origin in North America, brought as contaminants in forage seed lots. At present, both wild species are widespread in the central part of Argentina, and they extensively overlap the sunflower crop region and some areas devoted to seed multiplication. Flowering time and insect pollinators give opportunity for wild-crop hybridization. Genetic flow between cultivated and both wild species has been well documented in North America, where several wild *Helianthus* species are feral or weeds and originate hybrid swarms. Wild *H. annuus* easily hybridize with the cultivated sunflower because they constitute the same species. Crosses have been observed over 1000 m (Arias and Rieseberg, 1994, 1995; Whitton *et al.* 1997; Linder *et al.* 1998; Snow *et al.* 1998; Rieseberg *et al.* 1999a.)

Helianthus petiolaris also hybridize and introgress with the cultivated species, even when there are important reproductive barriers between them. The chromosomes of *H. petiolaris* and *H. annuus* differ at least in seven translocations and three inversions which affect 10 of their 17 pairs, therefore few hybrids are produced which tend to be highly sterile because of meiotic irregularities. However, backcrosses rapidly restore fertility in subsequent generations (Rieseberg *et al.* 1999a, b). Covas and Vargas López (1970) first described intermediate plants between *H. annuus* and *H. petiolaris* in Argentina. Hybrid status of similar plants was assessed by cytogenetic analyses (Ferreira, 1980) and isozyme markers (Carrera and Poverene, 1995) through crosses and progeny analysis. This situation has important biological and practical consequences, the former including homoploid hybrid species formation and the latter including a possibility of transgene spreading from genetically modified (GM) sunflower cultivars to wild or weedy populations (Ungerer *et al.*, 1998; Welch and Rieseberg 2002; Burke *et al.*, 2002; Snow *et al.*, 2003).

A remarkable fact is that in the last 25 years both wild *Helianthus* species found a favorable environment in central Argentina and they spread in semiarid habitats and sandy soils. According to our observations they have been established in at least seven provinces and they continue to expand. These wild species constitute a valuable source of genetic variation for incorporation of important characters into the cultivated sunflower, such as male sterility, pathogen resistance and herbicide tolerance. Sunflower is an important oil crop in Argentina, at present covering two millions ha with an annual yield of 3.8 millions tons. Transgenic crop production is also important in Argentina, being only second to the U.S. in the world. More than 80 sunflower GM varieties have been or are currently in field trials (CONABIA, 2003) and there is an interest of seed companies to begin with commercial production. The probability of gene transfer from cultivated sunflower towards wild populations is of concern because of the environmental impact that could be produced by the release of genetically modified sunflower cultivars and the potential risk of genetic modification of wild species.

We surveyed wild species distribution of populations in Argentina since 2000. Samples were collected at 140 sites for *H. petiolaris* and at 90 sites for *H. annuus*. Intermediate forms were found among typical wild plants in a number of locations. The goal of this work is to review some evidence about the cultivated sunflower and wild species hybridization in Argentina, and a number of experiments addressed to its quantification that are currently being evaluated. The results could be used to interpret genetic and ecological processes and to develop bio-safety criteria for the release of GM sunflower cultivars.

Variability and genetic structure of wild sunflower in Argentina

A wide array of biochemical and DNA markers are available for wild and cultivated sunflower (Carrera and Poverene, 1995; Rieseberg *et al.*, 1999; Carrera *et al.*, 2002, Paniego *et al.*, 2002). Genetic variability in *Helianthus annuus* and *H. petiolaris* was estimated using starch gel electrophoresis of eight isozymic loci. Wild plants were sampled in 20 populations of *H. annuus* and 22 populations of *H. petiolaris*, and seed isozymes were assayed. Mean number of alleles over all loci (A), proportion of polymorphic loci (P) and mean expected heterozygosity (H) were calculated. Genetic variation is currently being evaluated using SSR markers.

H. annuus diversity was similar to the levels reported for the center of origin (Rieseberg and Seiler, 1990; Cronn *et al.*, 1997), being A=1.6, P=0.37, and H=0.15. Similar levels of diversity have been found in Australia, where wild *H. annuus* was introduced for ornamental purposes (Dry, 1986). The diversity values were notably higher in *H. petiolaris* populations: A=1.94, P=0.63 and H=0.26. This fact can be attributed to multiple introduction events in Argentina. As we have already mentioned, the way through which *Helianthus* species were introduced is uncertain, but variability parameters provide evidence that these species have not been subjected to strong diversity reduction during the introduction process.

Total genetic variation (H_T) was similar in both wild species; however, they differed in the relative contribution of among- and within-population diversity. *H. annuus* displayed higher diversity values within the populations (H_S=0.31, G_{ST}=0.133) while *H petiolaris* values indicated that among-population diversity is the most important component in the hierarchical analysis (H_S=0.182, G_{ST}=0.319). The sampled populations were distanced from less than 1 km to more than 1000 km. Thus, some degree of genetic flow is likely to happen by pollen transfer but it would be restricted to closer population. In addition, these species have not noticeable seed dispersal abilities. *H. petiolaris* requires some conditions to germinate, namely disturbed and sandy soils, so environment discontinuities could also increase its genetic isolation. Moreover, high genetic differentiations can arise by founder effects. Genetic drift caused by a reduced number of individuals, a patchy distribution and agricultural practices (chemical weed control and tillage along road sides) can also explains differences among populations.

Natural hybrids occuring in central Argentina

Plants with intermediate morphological traits are seldom found in natural wild populations of both species and in cultivated fields in the central provinces of Buenos Aires, La Pampa, Cordoba and San Luis, where sunflower crop production is important. They also occur in the Western provinces of Mendoza and San Juan, near seed multiplication fields (Poverene *et al.*, 2002). According to the place where they were found and morphological traits, the rare intermediate plants could be attributed to different possible crosses:

1. Wild *H. annuus* female × crop male progeny was observed among wild populations on roadsides; wide leaves and heads, yellow discs and striate achenes were characteristic.
2. Crop female × wild *H. annuus* male offspring was found planted as cultivated seed, presumably due to a contamination of seed lots production; the traits were plant height, branching, small heads, and achene pubescence.
3. *H. petiolaris* female × crop male progeny was found among patches of wild populations on roadsides, fallow lots and along wire fences, near crop fields; characterized by plant height, head diameter, intermediate leaves, sterility and fasciation.
4. Crop female × *H. petiolaris* male offsprings were found in non-harvested sunflower lots or in second-year cultivated lots, near to wild *H. petiolaris* populations; slightly taller and branched plants, intermediate leaves and heads and sterility were the main characteristic traits. Situations 1 and 3 were by far the most frequently observed.

A number of intermediate plants collected in the wild were subjected to progeny tests, based on phenotypic, phenology and reproductive traits. Variation in progenies derived from 32 single, presumed hybrid plants, were compared with the variability of wild *H. annuus* and *H. petiolaris* accessions from eight different locations. Seedlings were raised in the greenhouse and transferred to field plots at the 4-6-leaf stage. Data were recorded on quantitative traits, such as seedling survival, plant height, branching, days to flowering, disc diameter, bract (phylaries) width, pollen viability, seed set and seed size, and on qualitative traits, such as leaf shape, presence of anthocyanin, disc color, male sterility, seed color and pattern (stripes, mottling) and seed pubescence. Morphological and fertility variation was tested through mean values, standard error, standard deviation, and range. Hybrid status of each maternal plant was assessed by segregation of qualitative traits among its progeny and increased variation of quantitative traits compared with that within accessions of both wild species. Most progenies confirmed the hybrid origin of their maternal plants, and a few of them seemed to be the advanced generation of volunteer plants. These results demonstrated that natural crosses between cultivated sunflower and both wild *Helianthus* species do occur, most probably because of pollen flow from crop to wild plants. Reciprocal crosses and flow among wild species seem to take place as well, but to a lesser extent.

Gene flow between sunflower and *Helianthus petiolaris*

Helianthus petiolaris was probably introduced before wild *H. annuus* in Argentina. It is much more abundant and widespread; populations are typically patchy and ephemeral, like in North America. Populations correspond to *H. petiolaris* ssp. *petiolaris*, according to Heiser (1961). Over the last six years, sunflower crops shifted towards the southwestern semiarid and sandy soils, as GM soybean crops became more popular under no-till system over the rich deep soils of the humid Pampa. At present, the sunflower crop area overlaps more than a half of the *H. petiolaris* distribution area, and wild plants grow side by side with sunflower crop fields. To assess hybridization between cultivated sunflower and this wild species, we screened progenies of *H. petiolaris* plants growing in populations up to 100m far from sunflower fields. We avoided small patches or single wild plants, because they should be presumably overexposed to crop gene flow. Bulked samples of seed were collected from wild heads exposed to pollen flow from the crop in 26 sites of three provinces. Subsets of seeds from each site were grown in the greenhouse and transplanted to field plots, raising almost 5000 plants. Hybrid plants were identified by intermediate morphology and data were collected on days to flowering, life cycle length, leaf size and shape, head and disc diameter, bract width, pollen viability (stained as Alexander, 1969) and seed set. Statistical parameters of mean value and standard deviation were calculated and tested for mean comparison. Morphological variation, phenology and fertility traits were also studied by principal components analysis and a hybrid index was calculated. Ten out of 26 sampled populations produced hybrid descendents, which were recognized by intermediate morphological traits and reduced fertility. Overall hybridization was of 1.3%; that is similar to the frequency of molecular markers introgression obtained by Rieseberg *et al.* (1999a). A single plant showed intermediate traits but a fairly good fertility and it might represent an advanced generation of a wild-crop hybrid or a backcross. Fertility is rapidly recovered in successive generations after interspecific hybridization in *Helianthus* (Rieseberg *et al.*, 1998). We are attempting to confirm these results through molecular marker (SSR) analysis.

Gene flow between cultivated and wild *Helianthus annuus*

Helianthus annuus, the wild ancestor from which the domesticated sunflower had been derived, is a diverse species in its center of origin and is also more variable than *H. petiolaris* in Argentina. The first report dates from 1974 in Entre Rios province, but we have reasons to suspect that it was a misleading description. It was not until 2000 when this species was reported for the main crop area (Poverene *et al.*, 2002). In fact, its distribution is more limited than that of *H. petiolaris* in the central provinces, but it grows on heavier, deeper soils in the eastern and western ends of central Argentina. There are already few places colonized by both wild species. Morphological traits of disc, bracts and seed size correspond to *H. annuus* ssp. *annuus* (Heiser, 1954). Gene flow from crop to wild populations probably accounts for the variability observed among and within populations, because the time elapsed since its introduction in the country has been too short to explain divergence due to natural selection or drift. However, it is difficult to assess crop-wild hybridization by phenotype examination. The predominant high branched, small-headed wild forms seldom show characters intermediate to the unbranched, large-headed cultivated sunflower. Nevertheless, we were able to identify some hybrid plants in the wild, as demonstrated through the progeny analysis experiment, and therefore we attempted to quantify gene flow in our ecological conditions. We employed an experimental design similar to that of Arias and Rieseberg (1994), using a sunflower cultivar homozygous for an acid phosphatase isozyme allele in a central plot, surrounded by small plots distanced from 3 to 1000 m of wild *H. annuus* plants. The Acp allele was absent in the wild population. The marker screening showed an overall frequency of 7% wild-crop hybrids. The highest gene flow rate was registered at 300 m. These results are useful to extrapolate from experimental populations to natural populations of wild sunflowers in the neighborhood of crop fields. Wild-crop hybrids produced 60-80% of seed set, thus demonstrating that introgression of neutral or beneficial crop transgenes into wild populations is feasible. Recently, Burke and Rieseberg (2003) and Snow *et al.* (2003) reported transgene escape and diffusion in wild sunflower populations, but pointed to the importance of quantifying fitness of each trait and ecological consequences of transgene spreading in wild populations.

CONCLUSIONS

Genetic variation studies in adventitious species provide useful information to trace back introduction and spread routes, to evaluate wild populations as genetic resources in crop improvement, and to fix bio-safety criteria concerning transgenic crop release. The two wild sunflower species in Argentina show a considerable amount of genetic variation, with values close to those found in their center of origin. Intermediate plants are most probably produced by reciprocal crossing between wild species and the cultivated sunflower. The hybrid status of such plants could be confirmed through segregation and variation among their progenies and we were able to quantify hybridization and gene flow for both wild species. It is likely that biological processes similar to those taking place in the center of origin could happen in central Argentina, in spite of the relatively recent introduction of both species. The high variability observed in wild sunflower in Argentina should be taken in consideration if transgenic cultivars are to be released, and studies on environmental impact in GM-wild sympatric zones would not probably apply to the whole range of distribution.

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ESPECIES SILVESTRES DEL GENERO *Helianthus* Y HIBRIDOS DE GIRASOL CULTIVADO Y SILVESTRE EN ARGENTINA

Resumen

Dos especies de *Helianthus* nativas de América del Norte se han naturalizado en Argentina, *H. annuus* ssp. *annuus* y *H. petiolaris* y crecen coincidiendo en un 50% con el área cultivada. La hibridación e introgresión entre estas especies silvestres y el girasol cultivado tienen importancia biológica y consecuencias prácticas, en la formación de híbridos homoploides y en la posibilidad de escape de transgenes desde cultivos de girasol genéticamente modificados (GM) a las especies silvestres o malezas. Se evaluó la variación morfológica e isoenzimática de las poblaciones silvestres. En diferentes localidades se encontraron plantas intermedias y algunas se sometieron a pruebas de progenie. La variabilidad encontrada dentro de las progenies fue comparada con la variabilidad presente en poblaciones silvestres de ambas especies. La mayor parte de las progenies mostró segregación para rasgos fenotípicos, fenología intermedia y niveles bajos de fertilidad, demostrando el origen híbrido de las plantas madres. A fin de cuantificar el flujo génico, se estudiaron progenies de poblaciones de *H. petiolaris* que crecían cercanas a cultivos de girasol. Las plantas híbridas fueron reconocidas por rasgos morfológicos y reducida fertilidad. El total de híbridos encontrados fue de 1%. La progenie híbrida en plantas de *H. annuus* silvestre fue identificada por un marcador isoenzimático. Se encontró una frecuencia promedio de hibridación del 7%. Estos resultados confirman que ocurre el flujo génico entre el cultivo y las especies silvestres de *Helianthus* lo cual tiene importancia en el manejo del cultivo y el impacto ambiental, si se aprobara la liberación de cultivos de girasol genéticamente modificados en Argentina.

ESPÈCES SAUVAGES DU GENRE *Helianthus* ET HYBRIDATION DU TOURNESOL SAUVAGE EN ARGENTINE

RÉSUMÉ

Deux espèces sauvages du genre *Helianthus* indigènes d'Amérique du Nord ont été naturalisées en Argentine, *H. annuus* ssp. *annuus* et *H. petiolaris*, et elles se répandent comme adventices en recouvrant environ 50% de l'aire cultivée. L'hybridation et l'introgression entre ces deux espèces sauvages et le tournesol cultivé ont une importance biologique et des conséquences pratiques dans la formation d'hybrides homoploïdes et dans la possibilité de dissémination de transgènes depuis des cultures de tournesol génétiquement modifiés (GM) vers les espèces sauvages ou envahissantes. Les populations sauvages ont été évaluées dans sa variabilité morphologique et des isozymes. Des plantes intermédiaires ont été trouvées dans plusieurs localités et on été soumises à des tests de descendance. La variabilité trouvée dans les descendances a été comparée avec la variabilité dans les populations sauvages des deux espèces. La ségrégation pour des caractères phénotypiques, la phénologie intermédiaire et des bas niveaux de fertilité ont été trouvés dans la plupart des descendances, en accord avec l'origine hybride des plantes mères. Avec le but de quantifier le flux de gènes ont a inclus l'étude des descendances de *H. petiolaris* qui se développaient proches des cultures de tournesol. Les plantes hybrides ont été reconnues par des caractères morphologiques et une fertilité réduite. La totalité d'hybrides trouvés a été de 1%. La descendance hybride dans les plantes *H. annuus* sauvages a été identifiée par la présence d'un isozyme spécifique du tournesol cultivé. On a trouvé une fréquence moyenne d'hybridation de 7%. Ces résultats confirment que le flux de gènes se produit entre les espèces cultivée et sauvages de *Helianthus*, ce qui est important pour la gestion des cultures et l'impact environnemental si on admet le lancement des cultivars de tournesol GM en Argentine.