

Genetic Architecture of Leaf Ecophysiological Traits in *Helianthus*

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Abstract

We investigated quantitative trait loci (QTLs) for several leaf chemistry traits in early-generation hybrids between *Helianthus annuus* and *Helianthus petiolaris*, the parental species of the ancient diploid hybrid sunflower species *Helianthus anomalus*, *Helianthus deserticola*, and *Helianthus paradoxus*. We grew individuals of a second-generation backcross (BC₂) toward *H. petiolaris* under optimum conditions in a glasshouse experiment. Trait values were measured once for each individual. In addition, genotypic data previously determined for each individual were employed for composite interval mapping of QTLs. We detected QTLs for leaf carbon concentration, leaf nitrogen concentration, leaf nitrogen per unit area, and photosynthetic nitrogen use efficiency. Leaf carbon isotope discrimination ($\delta^{13}\text{C}$) and leaf nitrogen isotopic composition ($\delta^{15}\text{N}$) were analyzed, but no significant QTLs were found for these traits. Interestingly, two neighboring loci explained a relatively large percentage of the variation in leaf nitrogen per unit area. This was notable because leaf nitrogen has been shown to strongly affect the fitness of early-generation sunflower hybrids in the *H. anomalus* habitat, and QTLs of large effect are expected to respond relatively quickly to selection. We speculate that the genetic architecture underlying leaf nitrogen may have facilitated the colonization of active desert sand dunes by *H. anomalus*.

Studies of the genetic architecture of ecologically important traits shed light on the evolution of those traits in natural populations. Studies of genetic architecture of complex traits are important because they can predict whether a phenotypic transition involved major leaps or occurred more smoothly (Burke et al. 2002). Also, alleles with large effects will fix more rapidly than will those of very small effect (Barton and Keightley 2002). An historical illustration of genetic architecture is the study by Beadle (1972) examining the inheritance of maize domestication traits. Using a primitive landrace of maize and a teosinte accession, Beadle observed the segregation of the maize and teosinte phenotypes at a 1:500 ratio, indicating that 4 or 5 genes of major effect were responsible for the bulk of changes from teosinte to maize (Beadle 1972). This demonstrated that the phenotypic transition from wild progenitor to primitive maize likely occurred in major leaps. This contrasts with sunflower, where domestication appears to have involved many small quantitative trait loci (QTLs), suggesting a smoother and more gradual transition to the domesticated form (Burke et al. 2002).

Phenotypic differences between wild sunflower species (*Helianthus*) also seem to be controlled by a large number of loci, each with a relatively small contribution to the phenotype (Kim and Rieseberg 1999; Rieseberg et al. 2003; Lexer et al. 2005). Two *Helianthus* species that have been the targets of previous genetic study, *Helianthus annuus* and *Helianthus petiolaris*, are of particular interest because they hybridized to form 3 stable, ancient, diploid hybrid species *Helianthus anomalus*, *Helianthus deserticola*, and *Helianthus paradoxus* (Rieseberg et al. 1990; Rieseberg 1991), which occupy extreme habitats compared with the parents: active sand dunes, dry desert floors, and brackish salt marshes, respectively. The range of phenotypes observed in the hybrid species is also more extreme than those of the parental species (Schwarzbach et al. 2001; Rosenthal et al. 2002).

Evolution of the hybrid sunflower species appears to have occurred quickly (Buerkle CA and Rieseberg LH, in review; Ungerer et al. 1998), and 2 of the hybrid species may have multiple origins (*H. anomalus*, Schwarzbach and Rieseberg 2002 and *H. deserticola*, Gross et al. 2003).

Additionally, phenotypes of the stable hybrid species are present in populations of early hybrids between *H. annuus* and *H. petiolaris* (Rosenthal, Rieseberg, and Donovan 2005). If the preadaptive phenotypic differences important for colonizing the extreme habitats of the hybrid species are controlled by multiple genes of small effect, then the rate of fixation of individual QTL alleles is likely to be slow and the sizes of parental chromosomal blocks in the hybrid species should be smaller than when loci of large effect control the phenotype (Barton and Keightley 2002). It seems paradoxical that most of the QTLs in *Helianthus* tend to be of small effect, but the hybrid species genomes seem to have stabilized relatively quickly (Buerkle and Rieseberg, in review; Ungerer et al. 1998). It may be the case, however, that variation in some of the traits that are important to the survival of early-generation hybrids in *Helianthus* are controlled by large-effect QTLs. Also, we know that pollen sterility QTLs, which also affect hybrid genomic composition (Rieseberg et al. 1996; Karrenberg et al. forthcoming), are controlled by major QTLs (Lai et al. 2005). Here, we examine leaf ecophysiological traits, some of which greatly affect fitness of plants in the wild.

Leaf nitrogen is likely to be ecologically important because it roughly estimates investment in nitrogen-rich photosynthetic enzymes, with investment per unit leaf area influencing photosynthetic capacity (Field and Mooney 1986). Foliar nitrogen is positively transgressive in the hybrid sunflower species *H. anomalus* (Rosenthal et al. 2002) and has been shown to impact survival in its native habitat (Ludwig et al. 2004). Despite being an important physiological trait, leaf nitrogen has only recently received attention in QTL analyses (Ishimaru et al. 2001; Hall et al. 2005; Takai et al. 2006; Weih et al. 2006). Other leaf chemistry traits measured in this study assess important aspects of plant physiology that are related to nitrogen allocation. Leaf carbon is a rough assessment of investment in carbon-rich structural molecules such as cellulose and correlates with construction cost of leaves (Nagel et al. 2002). Carbon isotopic ratio ($\delta^{13}\text{C}$) is often used as a proxy for water-use efficiency in C_3 plants (Farquhar et al. 1989; Ehleringer et al. 1992). Differences in leaf nitrogen isotopic composition ($\delta^{15}\text{N}$) for plants grown under similar conditions imply differences in nitrogen uptake or use (Evans 2001). Photosynthetic nitrogen use efficiency (PNUE) is the instantaneous rate of photosynthesis per unit leaf nitrogen and may be under selection in nutrient-poor or water-limited habitats (Field and Mooney 1986; Fredeen et al. 1991; Wright et al. 2002). Here we report the results of QTL analyses for these ecologically important, physiologically based leaf traits.

Materials and Methods

The plants used in this study were those of Rieseberg et al. (2003). The bulk of the phenotypic traits were summarized in the previous study, but the trait data reported here were not available at the time of the previous study. Second-generation backcross hybrids between *H. annuus* and *H. petiolaris* were generated as described previously by Rieseberg et al. (2003). Briefly, plants were grown from achenes collected from a population of *H. annuus* growing near Hanksville,

UT (ANN 1295) and a population of *H. petiolaris* growing near Page, AZ (PET 1277). F_1 seed was produced by crossing a single individual of *H. annuus* with a single individual of *H. petiolaris*. F_1 individuals were backcrossed with a second individual of *H. petiolaris* (PET 1277) to produce BC_1 seeds. Because of low seedset, each of the 38 BC_1 individuals was then backcrossed to a third *H. petiolaris* individual, producing BC_2 plants used for QTL analysis.

A total of 384 plants were germinated according to Schwarzbach et al. (2001) and transferred to 25-cm pots containing a 3:1 mixture of sand and baked clay (Turface, Profile Products, Buffalo Grove, IL) in the University of Georgia Plant Biology greenhouses in Athens, GA. Pots were watered to field capacity twice daily using an automated drip irrigation system, and nutrients were added as time-release fertilizer with macro- and micronutrients (Osmocote Plus, Scotts-Sierra Horticultural Products, Marysville, OH).

A fully expanded leaf was used to calculate specific leaf area (SLA = leaf area/leaf dry mass). Leaves were harvested in the morning when fully hydrated, and area was measured using a LI-COR 3100 area meter (LI-COR Biosciences, Lincoln, NE). Leaves were weighed after drying at 60 °C. Photosynthesis was measured starting 47 days after planting using a LI-COR 6400 portable gas exchange system between 10:30 AM and 04:30 PM. Chamber conditions were 360 ppm CO_2 , 26 °C, and 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation. Measured leaves were collected, dried at 60 °C, ground, and analyzed for nitrogen concentration (mg N/g biomass), carbon concentration (mg C/g biomass) (Carbo Erba NA 1500), and leaf carbon and nitrogen isotopic composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$).

PNUE was calculated using photosynthetic rate, leaf N, and SLA after Field and Mooney (1986). SLA was also used to convert nitrogen concentration on a weight basis into leaf nitrogen on an area basis.

Plants were genotyped for a total of 76 simple sequence repeat and 20 amplified fragment length polymorphism markers (for details, see Lexer et al. 2003). Markers were chosen from genetic maps of *H. annuus* (Burke et al. 2002; Tang et al. 2002) or *H. petiolaris* (Burke et al. 2004) to give relatively complete and even coverage of the linkage groups.

Phenotypic variables were corrected for block using a one-way analysis of variance implemented in JMP (SAS Institute, Cary, NC). The trait $\delta^{15}\text{N}$ was nonnormal, and those data were box-cox transformed in JMP. Coded marker data and corrected phenotypic data were imported into MAPMANAGER QTX version b20 (Manly et al. 2001). Linkage maps were assembled using the Kosambi mapping function as described previously (Lexer et al. 2003). Composite interval mapping (CIM) of the traits was carried out using QTL Cartographer (Basten et al. 1996). CIM was run at 2 cM resolution with 1000 permutations to determine log ratio score threshold value for the 0.05 level of significance.

Using MAPMANAGER QTX, data were analyzed for epistatic interactions for each of the traits with default $P = 0.00001$ significance threshold, which may be liberal given the number of possible pairwise comparisons between markers. The average effect and the partial r^2 for significant

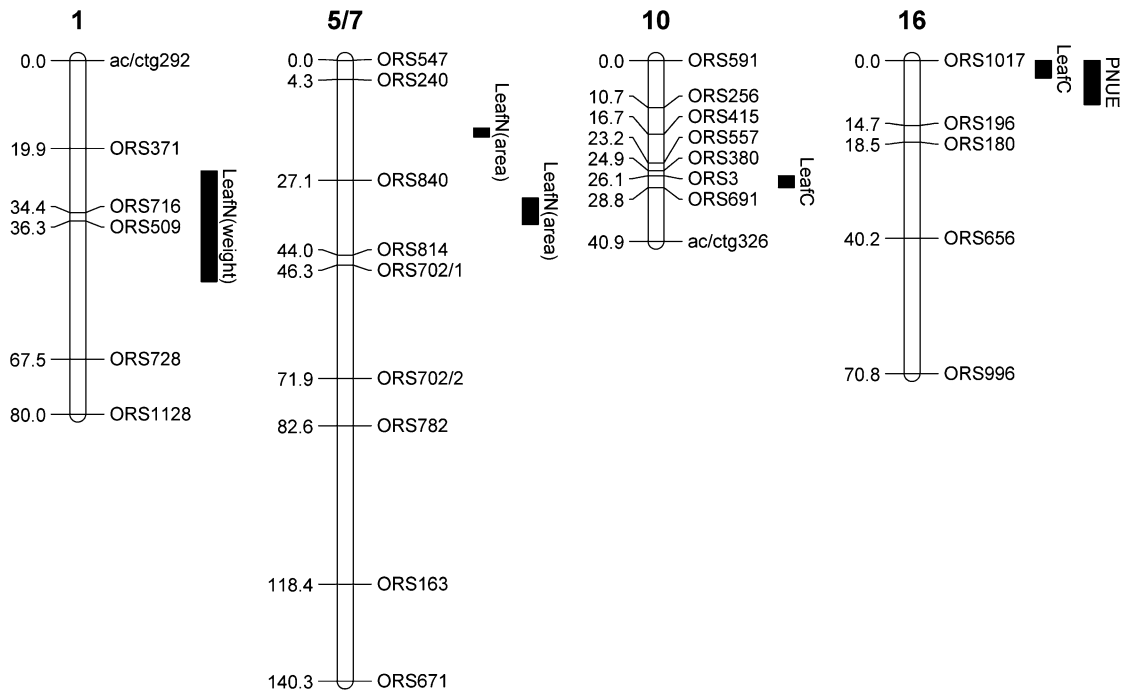


Figure 1. CIM results for each of the 7 leaf chemistry traits examined, 5 of which yielded significant QTLs. Significance threshold for each trait was determined through resampling the data, with 1000 repetitions, and chromosome walk speed was set at 2 cM. Shown are the 4 out of 17 linkage groups of the *Helianthus* genetic map where significant QTLs localized. Genetic distances (in cM) and marker names are provided.

interactions were estimated using the general linear model procedure in SAS version 8 (SAS Institute, Cary, NC). Partial r^2 was calculated as the sum of squares for the interaction term divided by the total sum of squares where variation in the trait was explained by the genotype at the 2 loci and their interaction.

Results

Significant additive QTLs were found for all traits, except $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (Figure 1 and Table 1). Especially noteworthy is the pair of QTL on linkage group V/VII, which together account for 24.6% of the variation in leaf nitrogen on an area basis.

Results for epistatic QTLs were less strong (Table 2). Only 2 traits had loci with significant interactions: leaf nitro-

gen on a weight basis and leaf carbon. For both, 2 significant marker pairs were found. The largest of these interactions explained $\sim 2\%$ of the variation in leaf carbon.

Discussion

For the majority of traits assessed in this study, the genetic architecture of leaf chemical traits appears to be largely influenced by multiple loci of small effect. A relatively small number of QTLs were detected, some of which were close to the detection limit ($r^2 = 0.04\text{--}0.06$), suggesting that there are a larger number of QTLs with small effects controlling these traits, with a subset being detected in our mapping study. One notable exception is leaf nitrogen on an area basis, where

Table 1. Composite interval mapping results

Trait (units)	Mean (SD)	Linkage group	Marker	Additive effect	LOD	r^2
Leaf C (g g^{-1})	0.4078 (0.0150)	10	ORS3	0.0129	3.6257	0.044
		16	ORS1017	0.0091	3.0715	0.038
Leaf N (mg g^{-1})	0.0583 (0.0063)	1	ORS716	0.0038	4.4215	0.056
Leaf N (mmol m^{-2})	136.87 (261.16)	5/7	ORS240	-27.74	2.7721	0.128
		5/7	ORS840	-27.88	3.1554	0.111
PNUE ($\text{mmol CO}_2 \text{ mol N}^{-1} \text{ s}^{-1}$)	289.3 (142.44)	16	ORS1017	-79.13	4.2487	0.116

Markers associated with significant QTLs in the CIM analysis were regressed against untransformed trait values, which were corrected for variation due to experimental block. r^2 values from this analysis are used to estimate the percentage of phenotypic variation explained by the QTLs detected in the CIM analysis. The additive effect is the average effect of the maternal (*Helianthus petiolaris*) genotype on the trait, and the mean and standard deviation (SD) of the mapping population are given.

Table 2. Epistasis results

Trait	Linkage group 1	Marker 1	Linkage group 2	Marker 2	Locus 1 LRS	Locus 2 LRS	Interaction LRS	Main effect 1	Main effect 2	Interaction effect	Partial r^2
Leaf C	10	ORS557	16	ORS1017	7.1	11.7	6.8	-0.0070	-0.0060	0.0167	0.0122
	10	ORS3	16	ORS1017	10.6	11.7	10.2	-0.0094	-0.0071	0.0209	0.0201
Leaf N (weight)	1	ORS716	9	ORS887	18	0.6	7.8	-0.0019	-0.0049	0.0030	0.0157
	1	ORS509	9	ORS887	6.9	0.6	18.9	-0.0012	-0.0037	0.0055	0.0131

Epistatic associations were determined in MAPMANAGER QTX at the recommended $P = 1.0 \times 10^{-5}$ level of significance. Provided are the chromosome number and marker for both loci, the log ratio scores (LRSs), and mean effects on the phenotype for both main effects and the interaction term. The partial r^2 value estimates the proportion of phenotypic variance that can be explained by epistasis.

2 QTLs account for nearly a quarter of the observed phenotypic variation. These results support the idea that leaf nitrogen is an important trait in the habitat of the stable hybrid species *H. anomalus* (Donovan et al. in review; Ludwig et al. 2004; Rosenthal, Ludwig, and Donovan 2005).

Using phenotypic selection analysis on early-generation hybrids between *H. annuus* and *H. petiolaris*, Ludwig et al. (2004) showed that some ecophysiological traits are under selection (sensu Lande and Arnold 1983) in the *H. anomalus* habitat. In other words, early-generation hybrids with higher foliar nitrogen were significantly more successful in the field, taking into account variation in correlated traits that also affected fitness. Because of the large effect of the 2 loci associated with leaf nitrogen, fixation of more fit genotypes could proceed more quickly (Barton and Keightley 2002). Also, because the variability in this interspecific cross is so large, the percentage of variation explained is likely an underestimate of the effect of the QTLs in natural populations of the *Helianthus* species used (Lexer et al. 2005). In natural populations of *H. anomalus*, the genomic regions where the QTLs localize are fixed for the *H. annuus* genotype as one would expect if there were heavy selection on foliar nitrogen in early hybrids (Rieseberg et al. 2003).

It is also noteworthy that no significant QTLs were found for $\delta^{13}\text{C}$ because the trait is known to be heritable and early-marker regression studies found significant genetic correlations in tomato (Martin et al. 1989). In other studies, Handley et al. (1994) localized $\delta^{13}\text{C}$ loci on chromosome 4 of barley, Mian et al. (1996) found 4 unlinked restriction fragment length polymorphism markers associated with $\delta^{13}\text{C}$ in soybean, and Juenger et al. (2005) detected 5 loci in *Arabidopsis thaliana*. However, work by Teulat et al. (2002) demonstrated that the QTLs for $\delta^{13}\text{C}$ are environment specific. Moreover, a larger number of QTLs were found in stressed than in unstressed environments. It is possible then that no significant QTLs for $\delta^{13}\text{C}$ were found because our plants were not stressed. It is also worth noting, as a general caveat, that a second-generation backcross population was used instead of an F_2 population in order to generate an appropriate number of progeny for QTL analysis. This may have hindered our ability to detect weak QTLs, but those loci are difficult to detect in any analysis (Mauricio 2001).

In addition to providing some information about an economically important crop species, QTL studies in *Helianthus* may provide information about the evolution of hybrid species. Of particular interest is whether additive effects explain

the bulk of the extreme phenotypic divergence seen in the hybrid sunflower species, as is suggested by previous work (Rieseberg et al. 2003) or whether nonadditive effects such as epistasis have a prominent role in trait evolution (Malmberg and Mauricio 2005). We detected 4 epistatic interactions, with the largest of these explaining only 2% of the phenotypic variation in leaf carbon. In every case, the effect of the interaction was in the opposite direction of the main-effect QTLs. This counterbalancing of the additive effects, coupled with the lack of significant interaction terms for most of the traits surveyed, lends support to the standing hypothesis that transgressive segregation accounts for the extreme phenotypes of the hybrid species (Rieseberg et al. 2003).

Further studies will focus on examining QTLs in early-generation hybrids planted in the field. It is likely that additional QTLs will be found when an analysis is done on field-grown plants (Paterson et al. 1991; Teulat et al. 2002).

Acknowledgments

The authors thank A. Howard, C. Gormally, S. Gevaert, and 2 anonymous reviewers for comments on an earlier draft of the manuscript. This work was supported by National Science Foundation grant IBN-0131078 to L.A.D. and National Institutes of Health grant GM59065 to L.H.R. This paper is based on a presentation given at the 2006 Annual Meeting of the American Genetic Association, "Genetics of Speciation," University of British Columbia, Vancouver, Canada, July 21–24, 2006.

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