Re-creating Ancient Hybrid Species' Complex Phenotypes from Early-Generation Synthetic Hybrids: Three Examples Using Wild Sunflowers

David M. Rosenthal,[1,*] Loren H. Rieseberg,[2,†] and Lisa A. Donovan[1,‡]

1. Department of Plant Biology, University of Georgia, Athens, Georgia 30602-7271;
2. Department of Biology, Indiana University, Bloomington, Indiana 47405-3700

ABSTRACT: Can the complex phenotypes that characterize naturally occurring hybrid species be re-created in early-generation artificial hybrids? We address this question with three homoploid hybrid species (*Helianthus anomalus, Helianthus deserticola, Helianthus paradoxus*) and their ancestral parents (*Helianthus annuus, Helianthus petiolaris*) that are phenotypically distinct and ecologically differentiated. These species, and two synthetic hybrid populations of the ancestral parents, were characterized for morphological, physiological, and life-history traits in greenhouse studies. Among the synthetic hybrids, discriminant analysis identified a few individuals with the multitrait phenotype of the natural hybrid species: 0.7%–1.1% were *H. anomalus*-like, 0.5%–13% were *H. deserticola*-like, and only 0.4% were *H. paradoxus*-like. These relative frequencies mirror previous findings that genetic correlations are favorable for generating the hybrid species' phenotypes, and they correspond well with phylogeographic evidence that demonstrates multiple natural origins of *H. deserticola* and *H. anomalus* but a single origin for *H. paradoxus*. Even though synthetic hybrids with hybrid species phenotypes are rare, their phenotypic correlation matrices share most of the same principal components (eigenvectors), setting the stage for predictable recovery of hybrid species' phenotypes from different hybrid populations. Our results demonstrate past hybridization could have generated hybrid species-like multitrait phenotypes suitable for persistence in their respective environments in just three generations after initial hybridization.

There are two contrasting mechanisms by which a new plant species can arise by hybridization (Grant 1981): allolyploid and homoploid hybrid speciation. Because hybridization must be initiated in sympatry, both forms of hybrid speciation require that reproductive barriers arise in the presence of gene flow. Allopolyploid speciation is straightforward because reproductive isolation is an instantaneous by-product of genome doubling. However, there is no assured means by which homoploid hybrid lineages become isolated from their parental species. Early authors (Stebbins 1957; Grant 1958; Templeton 1980) suggested that chromosomal rearrangements, ecological divergence, and spatial separation could isolate the hybrid neospecies, suggestions confirmed recently by computer simulation (McCarthy et al. 1995; Buerkle et al. 2000). Karyotypic divergence and spatial isolation are well documented in natural hybrid species (Gallez and Gottlieb 1982; Rieseberg 1991; Rieseberg et al. 1995; Hirai et al. 2000), and most hybrid species occupy habitats that are different or extreme compared with the parental species (Abbott 1992; Arnold 1997; Rieseberg 1997; Schwarzbach et al. 2001; Rosenthal et al. 2005).

The simulation models above assume that habitat differentiation must occur early in the speciation process (Buerkle et al. 2000). Hybridization provides a plausible mechanism for rapid niche differentiation between the parental species and neohybrids. Recombinant hybrids are expected to have more phenotypic variation than either parent (Falconer and Mackay 1996), and selection on these hybrids in different habitats can facilitate ecological divergence and adaptation to new environments (Stebbins 1959; Lewontin and Birch 1966). The rate at which the hybrids could respond to selection would be considerably

* Corresponding author. Address for correspondence: Portland State University, Department of Biology, P.O. Box 751, Portland, Oregon 97207; e-mail: drosen@pdx.edu.

† E-mail: lriesebe@indiana.edu.

‡ E-mail: donovan@plantbio.uga.edu.

enhanced if recombinant individuals were generated that possess multitrait phenotypes that are "preadapted" to adjacent open or underutilized niches. Indeed, this might be critical, given the need for "instant isolation" early in the hybrid speciation process (Buerkle et al. 2000). We explore this hypothesis for two reciprocal synthetic hybrid populations. We demonstrate that early-generation recombinant hybrids may be generated that have multitrait phenotypes similar to stable hybrid species.

Extreme phenotypes are often reported in natural and synthetic hybrid populations and could aid in the ecological isolation of hybrids. For example, three natural hybrid sunflower species, *Helianthus anomalus*, *Helianthus deserticola*, and *Helianthus paradoxus* have distinct multitrait phenotypes and occupy very different habitats (Heiser et al. 1969; Rosenthal et al. 2002, 2005; Welch and Rieseberg 2002*a*). Many of the traits that distinguish these species are extreme relative to parental values, and phenotypic selection experiments indicate that some are favored in their native habitats (Lexer et al. 2003*b*; Gross et al. 2004; Ludwig et al. 2004). The extreme or "transgressive" phenotypes often observed in recombinant hybrid populations (Devicente and Tanksley 1993) are primarily due to complementary gene action; alleles that have opposing effects within species (i.e., plus and minus alleles) are combined in segregating hybrids (i.e., F_2 or BC_x), thereby generating phenotypes that exceed those of the parental species (Rieseberg et al. 1999*b*). For example, suppose both parental species contain two alleles that increase plant height (plus alleles) and two that decrease plant height (minus alleles). Hybrid genotypes that contain all four plus alleles or all four minus alleles would be considerably taller or shorter, respectively, than their parental species. Transgressive segregation is less frequently caused by the nonadditive interaction of alleles between loci or epistasis, or the nonadditive interaction of alleles within a locus or overdominance (Devicente and Tanksley 1993; Monforte et al. 1997).

Recently, Rieseberg and colleagues demonstrated that transgressive segregation and complementary gene action underlie ecological transitions and adaptation in hybrid sunflower species (Rieseberg et al. 1999*b*, 2003; Lexer et al. 2003*a*, 2003*b*). Using a second generation backcross of *Helianthus annuus* × *Helianthus petiolaris* (BC_2PET), the authors were able to re-create many (but not all) of the extreme traits observed in the natural hybrid species. So how did the remaining traits arise? One possibility is mutational divergence after hybrid speciation, but this hypothesis would increase the theoretical difficulties associated with the process (Buerkle et al. 2000). Alternatively, traits that could not be recovered in the BC_2PET might be generated in a reciprocal backcross population. We an-

alyze a reciprocal backcross population and reveal how different genetic backgrounds affect trait expression.

Recombination following hybridization can increase or decrease correlations among traits in natural and synthetic hybrids when compared with the parental taxa (Clausen and Hiesey 1958; Grant 1979; Murren et al. 2002), but the extent to which hybridization alters phenotypic correlations is poorly understood. Phenotypic correlations generally reflect the underlying genetic correlations in plants and animals (Cheverud 1988; Waitt and Levin 1998). Among-population covariance of genetically determined traits may arise because of underlying developmental, functional constraints and/or in response to selection (Schlichting and Pigliucci 1998; Pigliucci 2003). Either way, multivariate genetic correlations play a critical role in how populations respond to selection (Lande 1979; Lande and Arnold 1983; Arnold 1992), particularly for hybrid populations where linkage disequilibrium is likely to be very strong. Because of high levels of linkage disequilibrium, phenotypic evolution of early-generation hybrids is likely to be more constrained by phenotypic correlations than that of their parental taxa (Murren et al. 2002). If hybridization is accompanied by consistent genomic changes, as has been demonstrated in annual sunflowers (Rieseberg et al. 1996, 2003), then we expect synthetic hybrids to exhibit consistent changes in phenotypic correlations. We examine the phenotypic correlation matrices between early-generation synthetic hybrids to understand the degree to which hybridization itself affects phenotypic correlations.

Recent greenhouse studies reported on phenotypic variation in the three hybrid species, their two parents, and one synthetic backcross (BC_2PET; Rosenthal et al. 2002; Rieseberg et al. 2003). Here we perform a detailed assessment of 43 morphological, physiological, life-history, and transgressive trait expressions in a reciprocal backcross (BC_2ANN) and compare our findings with trait expression in the BC_2PET population reported earlier (Rieseberg et al. 2003). Combining these data sets, we have information for 43 traits in two reciprocal backcross populations, the parents of which are known to have produced three hybrid species. This provides us with a unique opportunity to ask three important and related questions. First, can we identify individuals in the synthetic hybrid populations with the multitrait phenotypes of the hybrid species? Second, how might these different genetic backgrounds (BC_2ANN vs. BC_2PET) differ in their propensity to produce individuals with the complex multitrait phenotypes that are similar to the hybrid species? Finally, can differences or similarities in the synthetic hybrids' phenotypic correlation matrices explain our ability to detect hybrid species-like multitrait phenotypes in one synthetic backcross versus the other?

Material and Methods

Study Species

The five *Helianthus* species analyzed are diploid (n = 17), self-incompatible annuals, all native to North America. *Helianthus annuus* and *Helianthus petiolaris* are widespread in the North American plains and western states and occupy mesic clay soils and drier sandy habitats, respectively (Heiser et al. 1969). Studies in present-day hybrid zones suggest that genotypes tend to be distributed according to habitats (Stebbins and Daly 1961; Rieseberg et al. 1999a; Carney et al. 2000). *Helianthus petiolaris*-like individuals tend to be found in higher, drier habitats with sandier soil, and *H. annuus*-like individuals are found in wetter areas. These species are the ancestral parents of three hybrid species: *Helianthus anomalus, Helianthus deserticola,* and *Helianthus paradoxus* (Rieseberg 1991; Rieseberg et al. 1991), all of recent homoploid hybrid origin. Current estimates for the time of origin are between 144,000 and 116,000 ybp for *H. anomalus* (Schwarzbach and Rieseberg 2002), 170,000 and 63,000 ybp for *H. deserticola* (Gross et al. 2003), and 208,000 and 78,000 ybp for *H. paradoxus* (Welch and Rieseberg 2002b). They are also ecologically differentiated, being restricted to desert sand dune, desert floor, and brackish marshes, respectively.

Plant Material

We collected achenes (one-seeded fruits) from one population of each species: *H. annuus* (Hwy. 24, 1/4 mile N of Hanksville, Wayne Co., UT; Rieseberg 1295); *H. anomalus* (Hwy. 160, 6 miles E of Mexican Water, Apache Co., AZ; Rieseberg 1282); *H. deserticola* (I-15, Frontage Rd., 6 miles E of Toquerville, Washington Co., UT; Rieseberg 1270), *H. paradoxus* (S side of I-40, 1/4 mile E of exit 85, Grants, Cibola Co., NM; Rieseberg 1370), and *H. petiolaris* (Hwy. 89, boundary of Glenn Canyon Recreation Area, Kane Co., UT; Rieseberg 1277). For each population, we derived achenes from 30–100 maternal plants collected at irregular intervals along a transect. We pooled the achenes from all heads from a given population and chose a random subset for propagation at the University of Georgia greenhouses. The analysis of a single population per species is justified because most genetic variation (i.e., G_{st}) is found within rather than among populations of annual sunflower species (Heiser 1954, 1961; Schwarzbach and Rieseberg 2002; Welch and Rieseberg 2002b; Gross et al. 2003). Phenotypic variation (i.e., Q_{st}), however, often has a larger among-population component (Spitze 1993; Lynch et al. 1999; Schluter 2000). Therefore, our data are likely to underestimate the total species phenotypic variation of both the parental and hybrid species.

We derived synthetic hybrid populations from progeny from one *H. annuus* (ANN1295: northern city limit of Hanksville, UT) and one *H. petiolaris* (PET1277: Hwy. 89, 10 miles S of Page, AZ). For the BC_2PET, we backcrossed one F_1 hybrid from this cross with another individual of the PET1277. Due to high F_1 sterility, only 38 BC_1 progeny were produced. We then backcrossed these individuals with a third individual from PET1277 to yield the BC_2PET (hereafter BC_2PET). The BC_2ANN were produced in a similar manner except that the F_1 individual was backcrossed twice with ANN1295.

We germinated achenes following the protocol of Schwarzbach et al. (2001). We placed 20 seedlings from each population in 25-cm-diameter plastic pots containing a 3 : 1 mixture of sand/fritted clay (Turface, Profile Products, Buffalo Grove, IL). This mixture does not contain any nutrients or NaCl, which allowed us to add them in controlled quantities. Pots were arrayed in a randomized block design in the greenhouses, with 10 blocks and two individuals of each species per block. Each block also included 40 individuals from the BC_2ANN population (see above for crossing design), which is the focus of an ongoing quantitative trait locus (QTL) study (L. H. Rieseberg, D. M. Rosenthal, J. L. Durphy, and L. A. Donovan, unpublished study). We watered plants twice each day and fertilized with a time-release microencapsulated complete fertilizer (Osmocote Plus, Scotts-Sierra Horticultural Products, Marysville, OH). In addition, 8 days after planting, we treated the plants with 5.0 mM NaCl twice weekly for 3 weeks because previous studies indicated that *H. paradoxus* responds to salt stress by accumulating sodium in its leaves (Welch and Rieseberg 2002a). The very dilute NaCl treatment was high enough to ensure detection of this response in *H. paradoxus* but low enough so that the remaining species were unaffected. Some plants died early in the experiment due to transplant shock and were excluded from analyses. In all, we analyzed data from 20 individuals of *H. annuus*, 18 of *H. anomalus*, 18 of *H. deserticola*, 19 of *H. paradoxus*, 19 of *H. petiolaris*, and 383 BC_2ANN. Note that multivariate analyses necessarily excluded a few individuals because data was missing for some traits.

Traits Measurements

Twenty-three morphological and six life-history traits were measured (table 1). Morphological traits included seedling, vegetative, floral, and plant architectural traits. Floral traits comprised phyllaries, which are leaf-like bracts that underlie flowering heads, and ligules, which are the single florets found on ray flowers. We measured flower characters on fully expanded mature flowers in which 75% or more of the florets were mature. We measured leaf char-

Table 1: Trait abbreviations

Abbreviation	Description	Units
Morphological traits:		
BRHGT	Stem height at first branch	mm
BRNUM	Branch number at harvest	(Count)
DISKDIA	Flower disk diameter	mm
HARHGT	Height at final harvest	mm
HGT1	Height 7 days after planting (week 1)	mm
LFAREA	Leaf area	cm^2
LFLEN	Leaf length	mm
LFSHAP	Leaf shape (length/width)	mm/mm
LFSUC	Leaf succulence (wet wt − dry wt/leaf area)	mg/cm^2
LFTOUG	Average leaf toughness	(Relative scale)
LFWDTH	Leaf width	mm
LIGLGTH	Ligule length	mm
LIGNUM	Number of ligules (ray flowers)	(Count)
LIGSHAP	Ligule shape (length/width)	mm/mm
LIGWDTH	Ligule width	mm
PEDLGTH*	Peduncle length	cm
PETLEN	Petiole length	mm
PHYLGTH	Phyllary length	mm
PHYNUM	Phyllary number	(Count)
PHYSHAP	Phyllary shape (length/width)	mm/mm
PHYWDTH	Phyllary width	mm
STEMDIA	Stem diameter at 2 cm above ground	mm
SLA	Specific leaf area	$cm^2\ g^{-1}$
Life-history traits:		
BUDDAY	Days until budding	Days
FLODAY	Days until first floret	Days
FLOMASS*	Flower biomass	g
FLRNUM	Flower number at 65 days after planting	(Count)
HGR	Height growth rate (height at week 6 − height at week 1)/days	$cm\ day^{-1}$
SHBIO*	Aboveground biomass	g
Physiological traits:		
B	Leaf boron concentration	ppm
Ca	Leaf calcium concentration	ppm
CI	Leaf intercellular CO_2 concentration	ppm
COND	Leaf stomatal conductance	$mmol\ m^{-2}\ s^{-1}$
DEL13C	Leaf carbon isotope ratio ($\delta^{13}C$)	‰
DEL15N	Leaf nitrogen isotope ratio	‰
K	Leaf potassium concentration	ppm
LEAFC	Leaf carbon	$g\ g^{-1}$
LFNAREA	Leaf nitrogen per unit area	$mmol\ m^{-2}$
LEAFNG	Leaf nitrogen per unit leaf mass	$mg\ g^{-1}$
LFNMOL	Mol of nitrogen per unit leaf mass	$mmol\ g^{-1}$
Mg	Leaf magnesium concentration	ppm
Mn	Leaf manganese concentration	ppm
Na	Leaf sodium concentration	ppm
P	Leaf phosphorus concentration	ppm
PHOTO	Leaf photosynthesis	$\mu mol\ m^{-2}\ s^{-1}$
PNUE	Photosynthetic nitrogen use efficiency	$mmol\ CO_2\ mol\ N^{-1}\ s^{-1}$

Note: Only traits measured for both BC_2 populations are used to compare correlations and create decision rules. Asterisk = traits not used in multivariate analyses, either because traits were not measured in both populations or there were inconsistencies in how they were measured between populations.

acters on a fully expanded nonsenescent leaf from each individual. We measured leaf area using a LI-COR 3100 area meter (LI-COR, Lincoln, NE) and estimated average leaf toughness with a push dynomometer (McCormick Fruit Tech, Yakima, WA). The six life-history traits included timing of developmental stages (BUDDAY and FLODAY), allocation to floral and vegetative traits (FLRNUM, FLOMASS, SHBIO), and height growth rate (HGR).

We also measured 17 physiological traits such as photosynthetic rate, water and nitrogen use efficiency, and nutrient status. Photosynthetic rate, stomatal conductance, and internal CO_2 concentration were measured on a single mature leaf from each plant using a LI-COR 6400 portable photosynthesis system (CO_2 concentration 360 ppm, air temperature 26°–28°C, photosynthetically active radiation 1,500 μmol m^{-2} s^{-1}). Previous measurements indicated that *Helianthus* did not exhibit midday stomatal closure (Schwarzbach et al. 2001); therefore, we measured plants between 10:30 A.M. and 4:00 P.M. Due to the large number of plants, measurements were made on several days, so all gas exchange data were covariate corrected for time and day (Dudley 1996). As in Schwarzbach et al. (2001), we used internal CO_2 concentration as an estimate of instantaneous water use efficiency (CI; Farquhar et al. 1989; Donovan and Ehleringer 1994). We determined seasonally integrated water use efficiency from stable carbon isotope ratios ($\delta^{13}C$) of dried leaf material (continuous flow mass spectrometry; University of Georgia Stable Isotope Soil Biology Laboratory), abbreviated DEL13C in this study. More negative $\delta^{13}C$ values indicate reduced integrated water use efficiency for plants grown in similar environments.

To detect differences in nutrient status and use, we oven dried a mature leaf and then analyzed a portion of the leaf for percentage of N and C by Dumas combustion with a Carlo Erba NA 1500 elemental analyzer (Milan, Italy). We calculated photosynthetic nitrogen-use efficiency (PNUE) as the ratio of photosynthesis and N content per unit area of leaf (Field and Mooney 1986). We analyzed acid extracts from leaf tissue collected the day after the final 5 mM NaCl application for B, Ca, K, Mg, Mn, Na, and P on an inductively coupled plasma-atomic emission spectrophotometer (ICP-AES, University of Georgia, Chemical Analysis Facility).
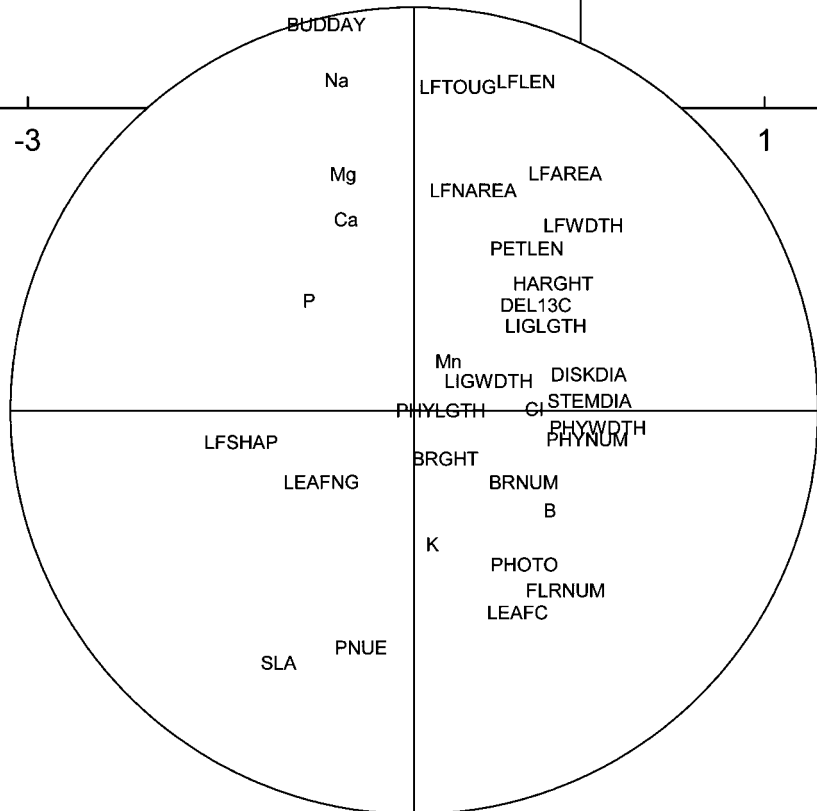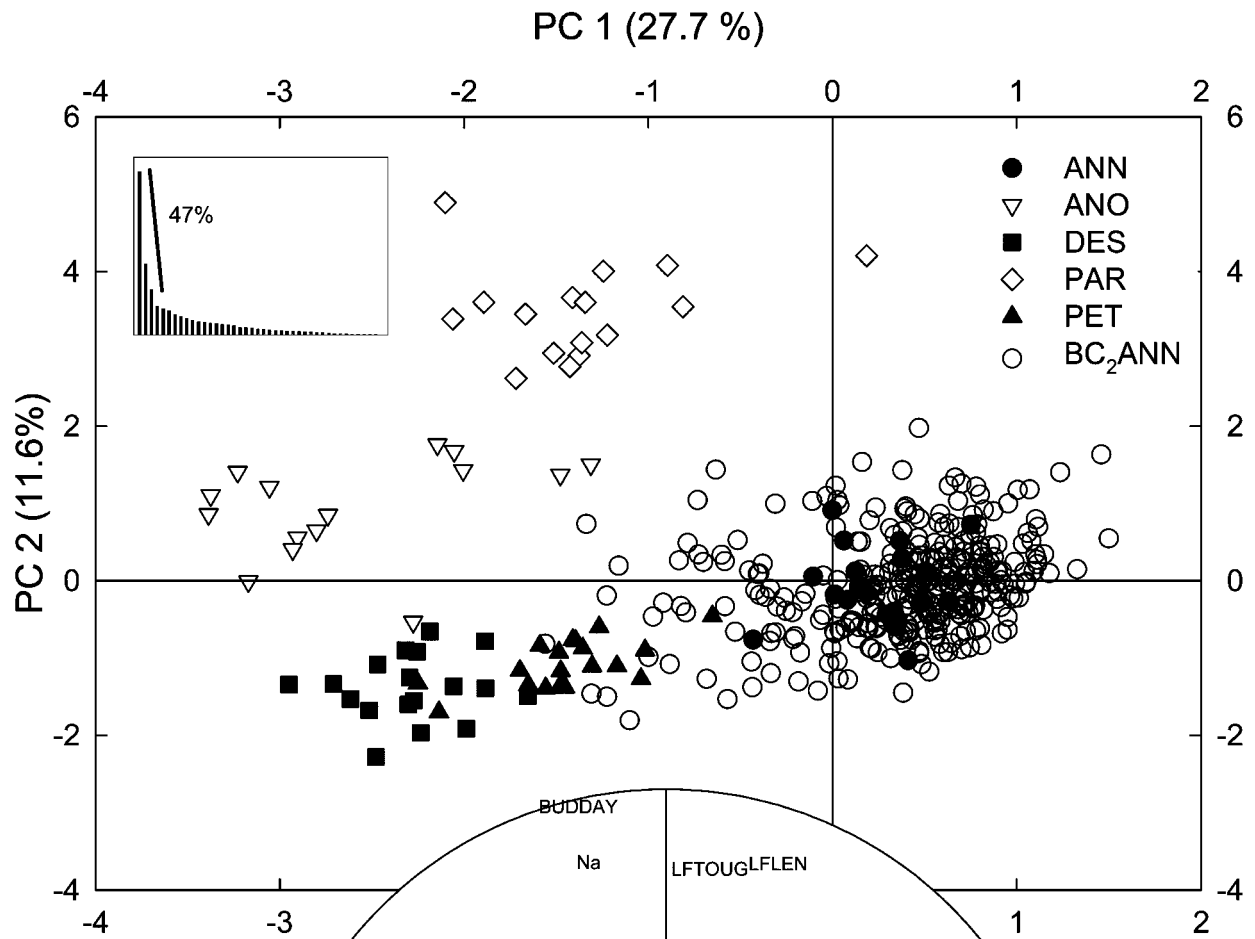
Statistical Analyses

We visualized differences and similarities in trait variation in the synthetic hybrids as well as the parental and hybrid species by principal component analysis on character-by-character correlations (Sokal and Rohlf 1995). We used discriminant analyses (PROC DISCRIM; SAS Institute 2001) to develop a discriminant criterion (rule) that would differentiate among five classes: the two parental species, *H. annuus* (ANN) and *H. petiolaris* (PET), and the three hybrid species, *H. anomalus* (ANO), *H. deserticola* (DES), and *H. paradoxus* (PAR). Only those traits measured on both synthetic hybrid populations ($n = 43$) were used when building decision rules (table 1). We used nonparametric methods ($k = 3$, nearest neighbor option) to avoid problems associated with deviation from multivariate normality. We calculated Mahalanobis distances (a multidimensional equivalent of Euclidian distance) from the pooled covariance matrix and used these distances to assign individual group membership. We did not have sufficient individuals to have an independent data set to test the decision rule, so we tested the decision rules by using the same calibration data set, which necessarily gives a biased estimate of the error rate. Therefore, to evaluate the robustness of the discriminant function, we implemented a jackknife resampling method (PROC DISCRIM, CROSSVALIDATE option). This process removed the observation being classified from the density estimation before determining the classification error rate (SAS 2001).

To determine if we could re-create a parental-like or hybrid species-like multivariate phenotype, we classified the synthetic hybrid populations (BC$_2$PET or BC$_2$ANN) according to the decision rule (discriminating the five species) generated in the above analysis. In this way we could identify synthetic hybrid individuals as belonging to one of six possible categories: ANN-like, ANO-like, DES-like, PAR-like, PET-like, or OTHER. Although the synthetic hybrid lineages were grown in separate experiments, individuals from all five species were grown in both experiments. Moreover, we replicated the experimental conditions as closely as possible so that any variation between experiments would affect taxa similarly.

Since we could not evaluate all possible discrimination rules, we performed a subset of analyses that were chosen

Figure 1: Principal components analysis (PCA) of 43 morphological, physiological, and life-history traits ($n = 43$) for the synthetic hybrid BC$_2$ANN, the parental species *Helianthus annuus* (ANN) and *Helianthus petiolaris* (PET) and the three hybrid species *Helianthus anomalus* (ANO), *Helianthus deserticola* (DES), and *Helianthus paradoxus* (PAR). The box in the upper left-hand corner shows the proportion of phenotypic variation explained by each component. The circle below the PCA illustrates how the traits contributed to separation along the two axes. Trait names closest to the edge of the circle indicate traits with higher loadings. Names that are closer to each other are more correlated on a given axis. When highly correlated traits also have similar loadings, they would plot to the same location. This happened in several cases, so some trait names were removed from the plot for clarity. For example, leaf succulence and leaf Na (*Na*) plot to the same coordinates, but only Na is plotted.

by considering both biological and statistical issues. The first discrimination rule based on all traits is the most conservative decision rule but is highly over parameterized. The second rule removes variables that are highly correlated such that when variables had r^2 values >0.7 or <−0.7, one of the two variables was removed. In the third rule, only transgressive traits are included, as reported here (see table A1 in the online edition of the *American Naturalist*) or in other studies (Rosenthal et al. 2002; Rieseberg et al. 2003). Finally, the fourth rule is based on a subset of traits selected in a stepwise discriminant analysis (PROC STEPDISC, SAS 2001). At each step, starting with no variables, the model is examined. Variables are added or removed from the model based on the significance level of an *F*-test from an ANCOVA ($0 < P < .25$ to add, $0 < P < .1$ to remove). The variables already chosen act as covariates, and the variable under consideration is the dependent variable. When a variable in the model that contributes least to the discriminatory power of the model, as measured by Wilks's λ, fails to meet the criterion to remain, it is removed. Otherwise, the variable not in the model that contributes most to the discriminatory power of the model is added. When all variables in the model meet the criterion to remain and none of the other variables meet the criterion to be added, the stepwise selection process stops (SAS 2001). The results of all four models are presented.

We compared the two synthetic hybrids' phenotypic correlation structures by comparing their phenotypic correlation matrices (**P** matrix), using a hierarchical principal component analysis (Flury 1988). This method is useful because matrices can share more detailed relationships than simply being equal or unequal (Flury 1988). We determined whether matrices share similarities at different hierarchical levels by testing a series of hypotheses about matrix similarity against the null hypothesis that they are unequal or "jump-up" approach (Phillips and Arnold 1999). At the lowest level of similarity, $p \times p$ matrices share one principal component (eigenvector) in common (PCPC 1). Hypotheses of increasing similarity are tested against the null hypothesis for up to $p - 2$ partial principal components. Matrices can share $p - 2$ principal components but have differing eigenvalues (CPC model). At the highest level of similarity (not equality), matrices differ

only by a constant (proportional model). In this case, matrices share all their principal components (or eigenvectors) but the eigen values differ by only a single constant. We also compare matrices using a model-building approach advocated by Flury (1988). The criterion for best model is not statistical significance, but the overall best fitting model is chosen based on Akaike's Information Criterion (AIC; Flury 1988). Akaike's Information Criterion balances the goodness of fit (log likelihood ratio) against the number of parameters used to fit the model. We used the Common Principal Component Analysis Program, developed by Patrick Phillips, University of Oregon, Eugene (program available at http://darkwing.uoregon.edu/~pphil/programs/cpc/cpc.htm).

We calculated post hoc pairwise means comparisons between the hybrid species and both parents using PROC MULTEST (SAS Institute 2001) and adjusted significance values for multiple comparisons using a sequential Bonferroni correction (Sokal and Rohlf 1995). We classified hybrid species traits as extreme (transgressive) when means significantly exceeded both parental values. We detected transgressive traits in the BC$_2$ANN population by comparing the number of BC$_2$ progeny that exceeded the high or low means of the parental populations by 2 or 3 SDs, with the numbers expected by chance.

Results

Phenotypic Variation and Transgressive Trait Expression

The BC$_2$ANN trait values and ranges were first analyzed individually and compared with the parental and hybrid species. The BC$_2$ANN traits encompassed the most of the range of both parents (table A1). The BC$_2$ANN was positively and/or negatively transgressive for 29 (63%) or 32 (70%) of the 46 traits measured. The low and high percentages depended on whether the observed number of BC$_2$ANN with extreme traits exceeded the low or high parental mean by 2 versus 3 SDs.

The BC$_2$ANN trait values also encompassed most of the range of hybrid species traits values. However, some hybrid species exceed even the BC$_2$ANN mean for several traits (table A1). For example, *Helianthus anomalus* had three of 16 (18.75%) extreme traits beyond the range of

Figure 2: Principal components analysis (PCA) of 43 morphological, physiological, and life-history traits ($n = 43$) for the synthetic hybrid BC$_2$PET, the parental species *Helianthus annuus* (ANN) and *Helianthus petiolaris* (PET), and the three hybrid species *Helianthus anomalus* (ANO), *Helianthus deserticola* (DES), and *Helianthus paradoxus* (PAR). The box in the upper left-hand corner shows the proportion of phenotypic variation explained by each component. The circle below the PCA illustrates how the traits contributed to separation along the two axes. Names closest to the edge of the circle indicate traits with higher loadings. Trait names that are closer to each other are more correlated on a given axis. When highly correlated traits also have similar loadings, they would plot to the same location. For example, bud date (*BUDDAY*) and leaf Na plot to the same coordinates, but only BUDDAY is plotted.
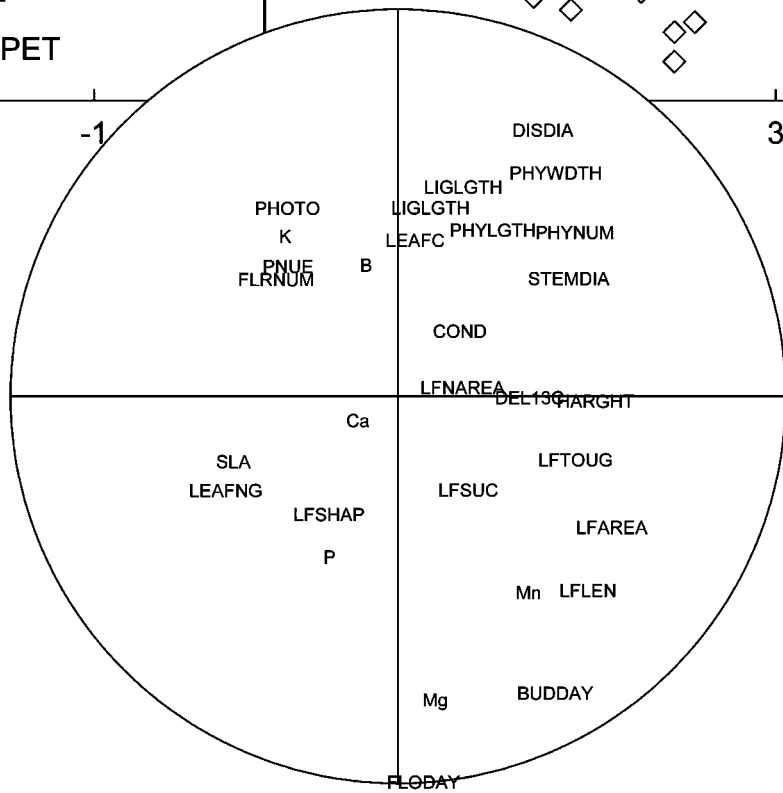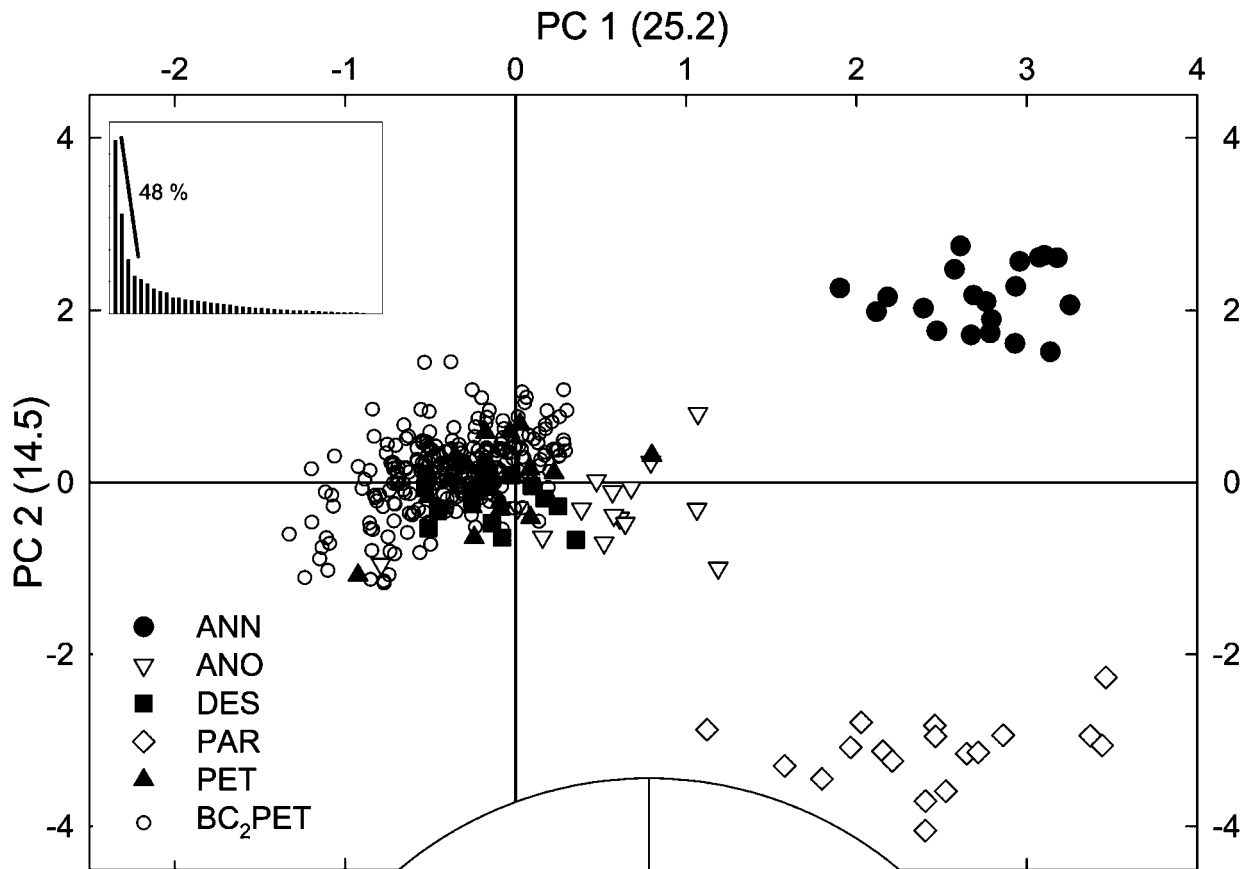
BC$_2$ANN: PHYSHAP, PHYWID, and STEMDIA. *Helianthus deserticola* had two of nine traits (22.2%) beyond the range of BC$_2$ANN: PHYSHAP and PHYWID. Finally, four out of 12 (33.3%) of *Helianthus paradoxus* traits exceeded the BC$_2$ANN range: LEAFTOUG, BUDDAY, FLODAY, and NA (see table 1 for definitions of the abbreviations used in this article).

The hybrid species were also extreme relative to the parents for numerous traits (table A1). *Helianthus anomalus* differed significantly from one or both parents for 31 traits, 16 of which were extreme. For *H. deserticola*, 30 traits were different from the parentals, 9 traits were extreme. *Helianthus paradoxus* was the most divergent with 33 significantly different traits, 13 of which were extreme (table A1).

Identifying and Re-creating Complex Multitrait Phenotypes

The multitrait phenotype of BC$_2$ANN was first compared with the five species. When all traits for BC$_2$ANN, the two parents, and the three hybrid species are considered simultaneously, the first three principal components of the PCA account for 47.2% of the variability in the data set (fig. 1). The BC$_2$ANN is highly variable; not surprisingly, much of the variation in BC$_2$ANN is explained by the same traits as in the recurrent parent *Helianthus annuus*. More importantly, there are several BC$_2$ANN individuals whose trait variation more closely resembles that of *Helianthus petiolaris*, *H. deserticola*, and even *H. anomalus*. However, no BC$_2$ANN individuals appear close to *H. paradoxus* (fig. 1). The BC$_2$PET population is similarly variable when compared to the five species, with the majority of the variation in BC$_2$PET explained by the same traits as in the recurrent parent, *H. petiolaris* (fig. 2). Moreover, BC$_2$PET appears to have even more individuals that resemble *H. petiolaris*, *H. deserticola*, and *H. anomalus* than BC$_2$ANN.

When the synthetic hybrids are compared to each other, the first principal component of the PCA with only BC$_2$ANN and BC$_2$PET accounts for nearly 43.2% of the variation in the data set (fig. 3). Morphological (size/shape) and physiological traits were important in explaining the variation between the synthetic hybrid populations. For example, BC$_2$ANN individuals have strong positive loadings for DISKDIA, PHYNUM, and LFAREA in the first principal component. In contrast, the BC$_2$PET population has strong negative loadings for PHYSHAP, LFLGTH, LFWDTH and PHOTO in the first principal component. Note that other principal components did not differentiate the synthetic backcross hybrid populations into distinct groups and are not shown.

Re-creating Hybrid Species' Multitrait Phenotypes from Early-Generation Hybrids

Discriminant analyses of the two parental and three hybrid species were used to construct classification rules that we applied to each of the synthetic hybrid populations. The results for the classification of early-generation hybrids make sense when we look at the numbers classified into the recurrent parent. For example, on average, 88% (83–97) of the BC$_2$ANN were placed into *H. annuus*, and 83% (79–92) of BC$_2$PET were placed into *H. petiolaris* (table 2). Not surprisingly, fewer synthetic hybrids were classified into the nonrecurrent parent phenotype. The BC$_2$ANN were classified as *H. petiolaris* about 10.1% (1.2–15.6) of the time, while none of the rules classified BC$_2$PET individuals as *H. annuus*-like.

Hybrid species-like phenotypes were much rarer among the synthetic hybrids than parental phenotypes, and their relative scarcity differed depending on the synthetic hybrid lineage. When analyzing the BC$_2$ANN population, *H. anomalus*-like individuals were recovered in all analyses about 0.7% of the time. *Helianthus deserticola*-like and *H. paradoxus*-like phenotypes were somewhat less common (0.5% and 0.4%). Unlike the other two hybrid species, *H. paradoxus*-like individuals were absent according to two of the four models (table 2). Overall, the decision rule error rates were low and similar (0%–1.9%) for all BC$_2$ANN decision rules. Classification error rates were higher because of ambiguity in assigning synthetic hybrids to parental classes; that is, most of the ambiguous cases were classified as part *H. annuus* and part *H. petiolaris*.

None of the rules classified BC$_2$PET individuals as *H. annuus*-like. *Helianthus anomalus*-like phenotypes were identified about 1% of the time. *Helianthus deserticola*-like phenotypes were much more common: on average, 13% of BC$_2$PET could be classified as *H. deserticola*-like. *Helianthus paradoxus*-like individuals were not observed in BC$_2$PET, regardless of the classification rule. Decision rule

Figure 3: Principal components analysis (PCA) of 43 morphological, physiological, and life-history traits for the synthetic hybrid BC$_2$ANN and BC$_2$PET. The box in the upper left-hand corner shows the proportion of phenotypic variation explained by each component. The circle below the PCA illustrates how the traits contributed to separation along the two axes. Names closest to the edge of the circle indicate traits with higher loadings. Trait names that are closer to each other are more correlated on a given axis. When highly correlated traits also have similar loadings, they would plot to the same location. This happened in several cases, so some trait names were removed for clarity. For example, flower number and leaf area (*LFAREA*) plot to virtually the same coordinates, but only LFAREA is plotted.
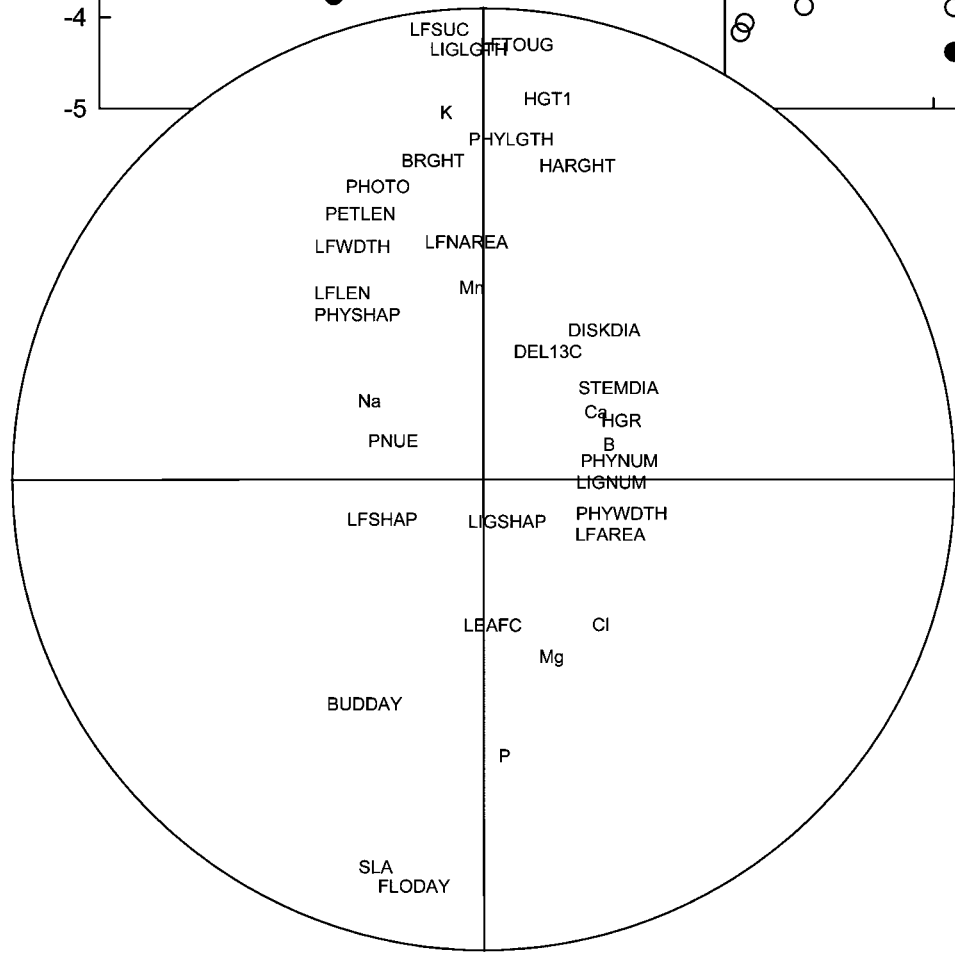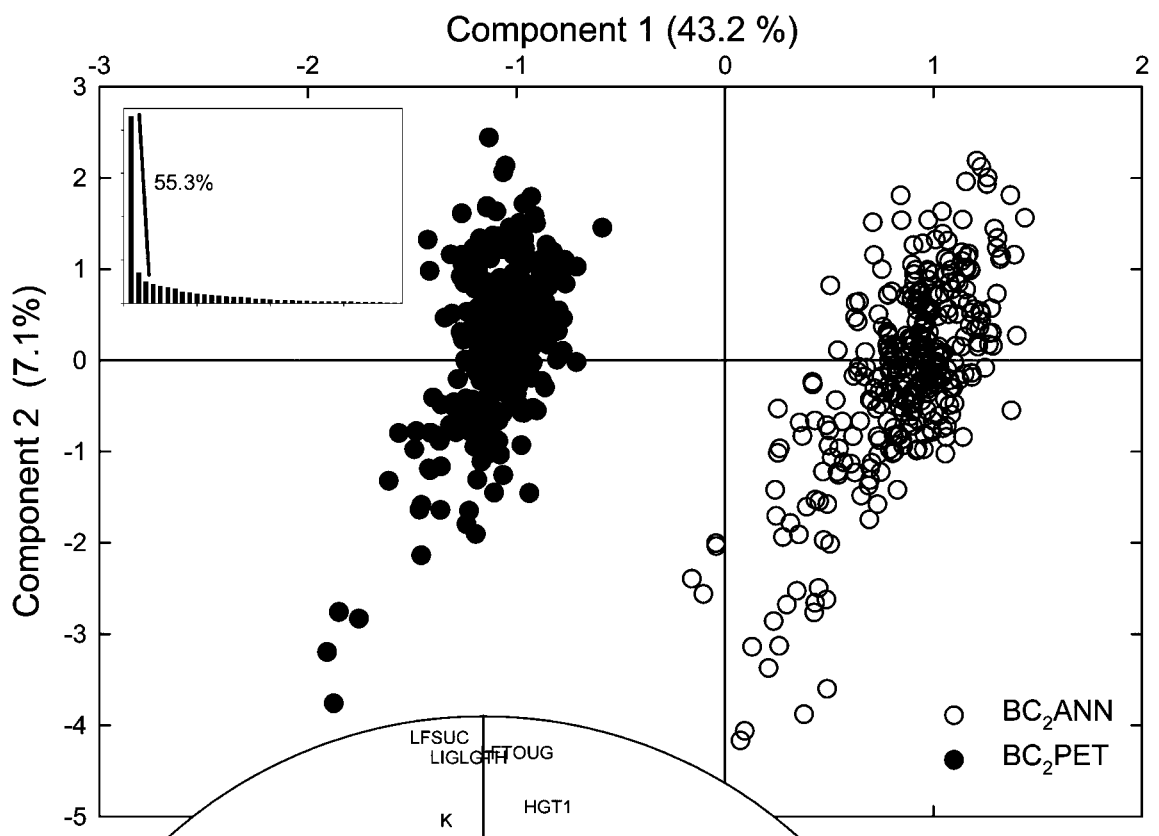
error rates were very low, and again, classification error rates were slightly higher. Here ambiguity was associated with BC$_2$PET cases that were petiolaris-like and/or deserticola-like. Nonetheless, BC$_2$PET were classified as *H. deserticola*-like unambiguously more than 50% of the time.

Phenotypic Correlations Matrices (**P** Matrix) in Synthetic Hybrids

Visual comparisons demonstrate that our first two testable hypotheses, matrix equality and proportionality, are rejected (fig. 4). If the matrices were identical, the points in figure 4 would all be on the dashed line (1 : 1). Similarly, if the matrices were proportional, the points would be distributed along another straight line that passes through the origin but with a different slope (e.g., Arnold 1992). Note that the regression intercept is very close to, but significantly different from, 0 ($t = 5.24$, df $= 901$, $P < .0001$). We tested the subsequent (lower) steps of matrix similarity using a hierarchical approach (Flury 1988). The jump-up approach indicates that the correlation structure of the synthetic hybrids share all their principal components in common and differ only in their eigen values (full CPC model; table 3). A model-building approach reveals a slightly different and more conservative estimate of similarity, with the best model being CPC (36) (table 4).

Discussion

Our results demonstrate that complex multitrait phenotypes of three divergent hybrid species can be detected in two populations of early-generation hybrids. The detection of these phenotypes in early-generation hybrids coupled with hybrid species associated with distinct and divergent habitats suggests that hybridization could have generated individuals with phenotypes suitable for persistence in their respective environments. Interestingly, it appears that the frequency with which we can recover hybrid species-like phenotypes in synthetic hybrid populations is related to the estimated number of origins of a given hybrid species.

We identify hybrid species-like phenotypes in both of the synthetic hybrid populations with four different models. The overall similarity of BC$_2$PET and the reciprocal backcross BC$_2$ANN phenotypic covariance matrices suggest that underlying genetic constraints (correlations) probably aid in the formation of the hybrid species complex multitrait phenotypes. The consistency with which hybrid species phenotypes can be detected in BC$_2$ANN and BC$_2$PET in this study corroborates recent work (Rieseberg et al. 2003), demonstrating that genetic correlations in BC$_2$PET could aid in re-creating some of the hybrid species' phenotypes.

Trait Expression in BC$_2$ANN and BC$_2$PET

When considering traits individually, the proportion of BC$_2$ANN individuals that had transgressive trait values (63%–70%) is slightly higher than those reported for the reciprocal artificial BC$_2$PET population (Rieseberg et al. 2003) and very high when compared with other studies of wild plants. For example, a survey of 131 studies of segregating hybrids in plants reported that only 58% of traits examined were transgressive overall (Rieseberg et al. 1999*b*). Transgressive segregation was reportedly much lower in wild populations than in domesticated lines (38% vs. 92%), and crosses between outcrossing and/or mixed mating species was lower than for selfing species (39% and 92%, respectively). When we considered only outcrossing wild plants, a mere 14% of the traits were transgressive (Rieseberg et al. 1999*b*). Clearly, both BC$_2$PET and BC$_2$ANN have a very high number of transgressive traits for a wild, outcrossing plant (Rieseberg et al. 1999*b*). The unusually high rate of transgressive traits in both of these synthetic populations attests to the potential for synthetic hybrids to have individuals with multitrait phenotypes similar to the natural hybrid species.

Extreme traits in the ancient hybrid species do not necessarily have to have arisen immediately following hybridization. For example, *Helianthus anomalus* and *Helianthus deserticola* have significantly fewer ray florets (LIGNUM) than parental species, yet BC$_2$PET and BC$_2$ANN have only positively transgressive individuals (i.e., individuals with phenotypic values greater than either parent). The most recent estimates place the origin of the hybrid species approximately 60,000 ybp (Gross et al. 2003); therefore, some phenotypic differences (or extreme traits) almost certainly arose through selection on mutations arising after hybrid speciation. The response of BC$_2$ANN and BC$_2$PET populations to selection in the native hybrid species' habitats was examined for each of the hybrid species (Lexer et al. 2003*b*; Gross et al. 2004; Ludwig et al. 2004). While those experiments did not measure selection on LIGNUM, they demonstrate that selection on synthetic hybrids in hybrid species' habitats favors the hybrid species' phenotypes in several instances (Gross et al. 2004; Ludwig et al. 2004).

Alternatively, it may be that some traits have other genetic or developmental constraints (Schlichting and Pigliucci 1998; Pigliucci 2003). It is interesting that the mean LIGNUM as well as associated floral traits (PHYNUM, DISKDIA) are virtually identical within the five species when compared between years (Rosenthal et al. 2002; this article). While it is not unusual that these floral traits covary, this is surprising given the variability we see among years for other traits. Their similarity from one year to the next suggests they are highly canalized and probably

Table 2: Summary of BC$_2$ANN and BC$_2$PET classifications based on four different discriminant rules

Rule type	ANN-like	ANO-like	DES-like	PAR-like	PET-like	Posterior probability error rates	
						Decision	Classification
BC$_2$ANN:							
Rule 1 ($n = 332$, traits $= 43$):							
n	296	1	2	0	33	…	…
%	89.2	.3	.6	0	10	.0	.048
Rule 2 ($n = 341$, traits $= 35$):							
n	283	3	1	1	53	…	…
%	83	.9	.3	.3	15.6	.015	.067
Rule 3 ($n = 336$, traits $= 21$):							
n	326	4	2	0	4	…	…
%	97	1.2	.6	0	1.2	.018	.0271
Rule 4 ($n = 342$, traits $= 25$):							
n	288	1	1	5	47	…	…
%	84.2	.3	.3	1.46	13.8	.0	.064
Means	88.35	.675	.45	.44	10.15	.008	.052
BC$_2$PET:							
Rule 1 ($n = 265$, traits $= 43$):							
n	0	4	48	0	213	…	…
%	0	1.5	18.11	0	80.4	.0	.085
Rule 2 ($n = 265$, traits $= 34$):							
n	0	1	18	0	246	…	…
%	0	.4	6.79	0	92.83	.0	.052
Rule 3 ($n = 295$, traits $= 18$):							
n	0	3	51	0	241	…	…
%	0	1.2	7.3	0	81.7	.012	.114
Rule 4 ($n = 266$, traits $= 28$):							
n	0	3	52	0	211	…	…
%	0	1.1	19.6	0	79.32	.0	.07
Means	0	1.05	12.95	0	83.56	.003	.08

Note: Rule 1 = all traits were used; rule 2 = highly correlated ($-0.7 > r^2 > 0.7$) traits removed; rule 3 = only traits transgressive for that backcross were used; rule 4 = traits selected based on stepwise discriminant analysis. Note that pedicel length, flower biomass, and shoot biomass were excluded from this and other multivariate analyses because they were not measured in both populations or because of inconsistencies in how they were measured between populations.

form a "correlation Pleiades" (Berg 1960). Such correlation and constraint should reduce the amount of variation we see for those characters in spite of hybridization.

Multitrait Phenotypes and Correlations

When considering all the traits simultaneously, some synthetic hybrids were classified as one of the three hybrid species. Overall, the classification results show that the complex multitrait phenotypes of the hybrid species can be re-created in early-generation hybrids. However, these are generally rare occurrences. For example, in all BC$_2$ANN cases, <2% of individuals were classified as similar to hybrid species. The proportion of BC$_2$PET recognized as hybrid species-like was higher (4.7%). However, the proportion of BC$_2$PET classified as hybrid species-like was radically skewed toward *H. deserticola* (7–18%). This

makes sense because *Helianthus petiolaris* and *H. deserticola* are similar phenotypically in many respects. The high proportion of *H. deserticola*-like hybrids in the BC$_2$PET population is also consistent with previous multivariate analyses that had difficulty differentiating between the two species (Rosenthal et al. 2002). Furthermore, *H. deserticola* trait values for flowering time, leaf size, and stem diameter are closer to BC$_2$PET than BC$_2$ANN when grown in *H. deserticola* habitat (Gross et al. 2004).

Given that we only evaluated two synthetic hybrid populations, the finding of even the occasional hybrid species-like individual is remarkable. Nonetheless, the predictions based only on the two crosses presented here underestimate the capability for *H. annuus* × *Helianthus petiolaris* hybrid lineages to yield hybrid species-like individuals. As noted earlier, hybrid zones are likely to have numerous individuals from several types of early and later generation
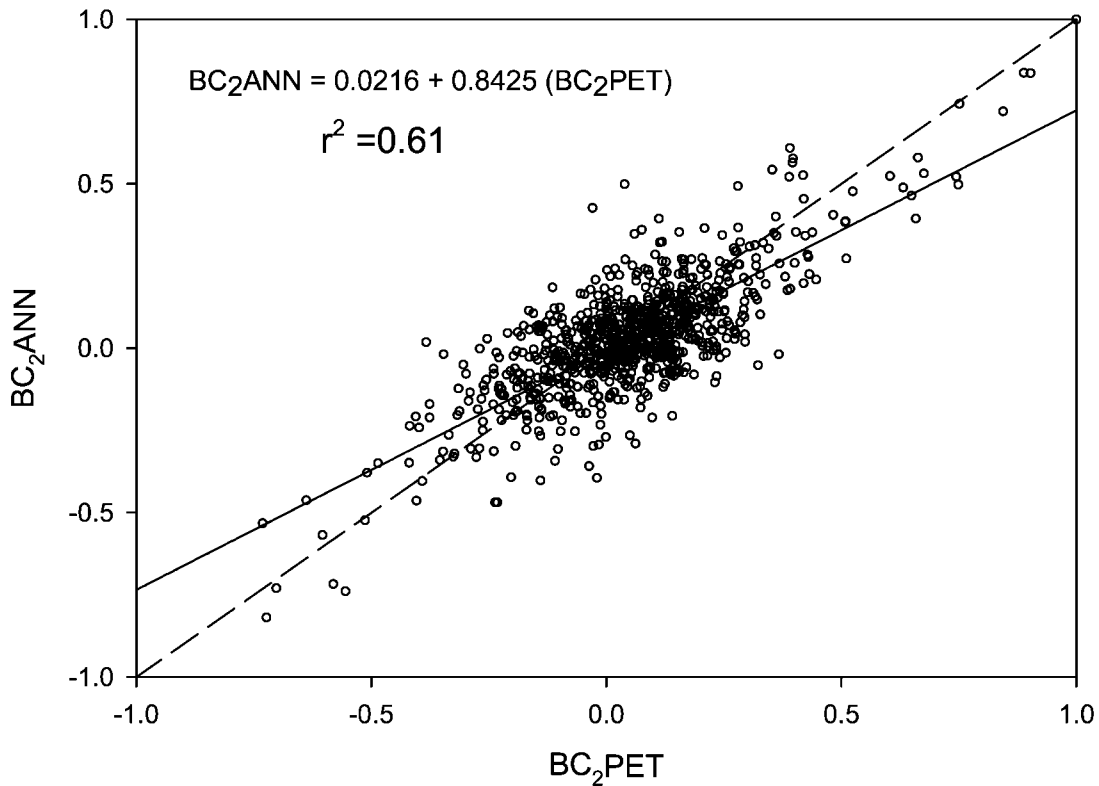
Figure 4: Scatterplot of all bivariate correlations for 43 traits for BC_2ANN (Y-axis) and BC_2PET. Correlations based on $n = 265$ individuals for BC_2PET and $n = 332$ for BC_2ANN. The solid line is the least squares regression line (slope and intercept are significantly different from 0, $P <$ .0001). The dashed line is the expected alignment of points if correlation matrices are identical (1 : 1 line).

recombinant lineages (Cruzan and Arnold 1993) with a greater array of possible phenotypes and genotypes. Moreover, the relatively small sample sizes used here for the species may not reflect the entire range of each species' phenotypic variation. Finally, the crossing design, necessary for the associated QTL mapping studies of BC_2PET (Rieseberg et al. 2003) and BC_2ANN (L. H. Rieseberg, unpublished data), is likely to underrepresent variation in either of the parental species. This implies that the discriminant criteria ultimately yielded a conservative estimate of hybrid species-like phenotypes.

Within and among population differences in phenotypic correlation matrices in general (i.e., integration in the broad sense) can be due to deterministic ecological and genetic factors in the short term and stochastic influences (genetic drift, mutation, and geneflow) in the long term (Waitt and Levin 1993; Armbruster and Schwaegerle 1996; Schlichting and Pigliucci 1998; Pigliucci and Hayden 2001). Past work suggests that both endogenous and exogenous selection (sensu Arnold 1997) largely control genomic composition in stabilized sunflower hybrids (Rieseberg et al. 1996, 2003; Lexer et al. 2003a) with ex-

ogenous selection having a slightly larger effect. The large size of parental chromosomal segments, consistent genomic composition, and tight linkage or pleiotropy among QTLs in synthetic or natural hybrid lineages (Rieseberg et al. 1995, 1996, 2003) implies that traits associated with those segments are likely to covary. The similarity of BC_2ANN and BC_2PET correlation matrices and the consistency with which we detected synthetic hybrids with hybrid species-like phenotypes reinforces the observations

Table 3: Flury hierarchy for the comparison of phenotypic correlation matrices for two synthetic hybrids (BC_2ANN and BC_2PET) using the jump-up procedure

Higher	Lower	χ^2	df	P
Equality	Unrelated	4,377.27	946	<.0001
Proportional	Unrelated	4,302.339	945	<.0001
CPC	**Unrelated**	**691.373**	**903**	**1**
CPC(1)–CPC(41)	Unrelated	…	…	1

Note: At each step in the hierarchy, a higher hypothesis is tested against the lower hypothesis that the matrices are unrelated. The bold type indicates the matrices share common principal component (CPC) structure.

Table 4: Flury hierarchy for the comparison of phenotypic correlation matrices for two synthetic hybrids (BC_2ANN and BC_2PET)

Higher	Lower	χ^2	df	P	χ^2/df	AIC
Equality	Proport	74.931	1	<.0001	74.931	4,377.27
Proport	CPC	3,610.965	42	<.0001	85.975	4,304.339
CPC	CPC(41)	3.488	1	.0618	3.488	777.373
CPC(41)	CPC(40)	1.209	2	.5464	.604	775.885
CPC(40)	CPC(39)	1.842	3	.6058	.614	778.676
CPC(39)	CPC(38)	71.215	4	<.0001	17.804	782.834
CPC(38)	CPC(37)	19.178	5	.0018	3.836	719.619
CPC(37)	CPC(36)	37.928	6	<.0001	6.321	710.441
CPC(36)	**CPC(35)**	**7.41**	**7**	**.3875**	**1.059**	**684.513**

Note: At each step in the hierarchy, the hypothesis labeled "Higher" is tested against the step below, "Lower." The best solution (in bold) under this model-building approach is indicated by the lowest value of Akaike Information Criterion (AIC). Note that comparison of CPC(34) to CPC(1) all have greater AIC and are omitted from the table for clarity.

that deterministic processes underlie the initial formation of hybrid species multitrait phenotypes. In spite of seemingly strong genetic and genomic constraints, the small but apparent differences in trait correlations and apparent phenotypes may result in different responses of BC_2PET and BC_2ANN to selection (e.g., Lexer et al. 2003*b*; Gross et al. 2004; Ludwig et al. 2004).

We note that the environmental/ecological factors can cause differences in the phenotypic (**P**) and genotypic (**G**) matrices (sensu Lande and Arnold 1983). Since the synthetic hybrids here were grown in the same greenhouses but in different years, it is possible some of the similarities/differences we see in trait correlations have an environmental basis (Begin and Roff 2001; Pigliucci and Hayden 2001). However, the close similarities in the correlation matrices and phenotypic differences are consistent with expectation for closely related synthetic hybrids (Begin and Roff 2003).

Phenotypic Divergence and Lineage Age

While BC_2ANN and BC_2PET individuals were most commonly classified as one of the parental species, *H. deserticola*-like individuals were identified by all the decision rules and from both reciprocal crosses. Recent surveys have revealed four different CpDNA haplotypes across eight *H. deserticola* populations (Gross et al. 2003). One was unique to *Helianthus annuus*, another to *H. petiolaris*, and two more were equally distributed among the parental taxa. Similarly, the 18 nuclear loci analyzed in the same populations were evenly distributed between the parental taxa. The frequency and repeatability of recovery of the *H. deserticola*-like individual reflects the probable multiple origins of *H. deserticola*. *Helianthus anomalus*-like individ-

uals were also identified by every decision rule and in both synthetic hybrid lines. Chloroplast data for this species revealed four haplotypes, two of which were common to both parents (Schwarzbach and Rieseberg 2002). Furthermore, crossing data and meiotic analyses revealed three different karyotypic forms, which were closely correlated in geographic distribution with chloroplast DNA haplotype. Analyses of genetic relationships among *H. anomalus* microsatellite loci were more difficult to interpret. Nonetheless, the most parsimonious explanation is that *H. anomalus* had multiple origins (Schwarzbach and Rieseberg 2002). The consistency with which we can recognize *H. anomalus*-like or *H. deserticola*-like individuals by discriminant analysis reflects their purported multiple origins. This is also consistent with earlier work demonstrating the consistency with which the genome of these species can be reconstructed (Rieseberg et al. 1996, 2003).

In contrast to *H. anomalus* and *H. deserticola*, synthetic hybrids resembling *Helianthus paradoxus* were considerably more difficult to find in the BC_2ANN population and were never identified in the BC_2PET populations. Unlike the other two hybrid species, a survey of *H. paradoxus* populations revealed only one CpDNA haplotype, which was only found in *H. annuus* populations. In fact, when CpDNA and nuclear microsatellite data are viewed together, they are consistent with a single origin for *H. paradoxus* (Welch and Rieseberg 2002*b*). The difficulty in recognizing *H. paradoxus*-like individuals in general (when compared to *H. deserticola* and *H. anomalus*) is consistent with a single origin hypothesis. The apparent absence of any *H. paradoxus*-like plants in the reciprocal backcross with an *H. petiolaris* maternal individual is also noteworthy in light of the fact that it is fixed for an *H. annuus* haplotype.

Conclusions

Hybridization undoubtedly played an important role in the adaptive evolution of annual sunflowers. An important caveat is that we do not know the habitats, or the particular locations, where the initial hybridization events took place. However, re-creating the species' complex phenotypes, each with a distinct and divergent habitat, suggests that hybridization today could generate individuals with phenotypes suitable for persistence in their respective environments. This is particularly encouraging since the initial hybridization event between *H. annuus* and *H. petiolaris* occurred at least 60,000 ybp (Schwarzbach and Rieseberg 2002; Welch and Rieseberg 2002*b*; Gross et al. 2003). Overall, the implication of this work is that several natural hybridization events similar to the two we generated are likely to have produced individuals with phenotypes suit-

able for colonizing habitats divergent from either parent within three generations.

Acknowledgments

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