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# Hybrid zones as a tool for identifying adaptive genetic variation in outbreeding forest trees: lessons from wild annual sunflowers (*Helianthus* spp.)

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## Abstract

The identification and study of adaptively important genes in forest trees represents a formidable challenge because of their long generation spans. In annual or perennial herbs, formal genetic studies can be employed to identify the quantitative trait loci (QTLs) and/or candidate genes that underlie important traits, and the segregating populations can be transplanted into natural populations to measure the strength and direction of selection. However, the application of these methods to forest trees is difficult, because the creation of appropriate genetic material is extremely time-consuming in long-lived, woody plants, and lifetime fitness estimates are difficult or impossible to obtain. Although QTL mapping should in principle be feasible in wild intraspecific populations (as an alternative to artificial crosses), this approach is less likely to be successful in trees because LD (linkage disequilibrium) will decay quickly in large outbreeding plant populations. Within the present paper, we discuss a modified approach based on natural hybrid zones. We describe the use of wild annual sunflowers (*Helianthus* spp.) as a model for exploring the hybrid zone approach. Transplanted experimental hybrids allowed us to assess the adaptive value of individual chromosomal blocks in nature, and data on natural *Helianthus* hybrids suggest that similar approaches are possible in natural hybrid zones. Our results allowed us to test the role of hybridization in the origin of ecological divergence in wild sunflowers. In addition, they have practical implications for identifying adaptively important genes or QTLs in trees. This is exemplified by three temperate forest taxa, *Populus* (poplars, aspens, cottonwoods), *Fraxinus* (ash), and *Quercus* (oak). All three are diploid and important genomic tools are under development. Moreover, all three offer extensive hybrid zones whose likely age can be inferred from fossil data. Age data enables estimates of the size and frequency of chromosomal blocks in hybrids, thereby providing guidance in designing marker-based experiments. We predict that natural hybrid zones will be valuable tools for identifying the QTLs and/or candidate genes responsible for adaptive traits in forest trees.

### Keywords

Hybrid zones; Admixture; Introgression; Adaptation; QTL; Species barriers

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## 1. Introduction

The importance of adaptive genetic variation is widely recognized in forest ecology and management. The longevity and wide geographic distributions of tree species place a premium on genomic diversity because of the need to adapt to a wide range of site conditions (Hamrick and Godt, 1989; Isabel et al., 1995; Hamrick and Nason, 2000). In addition to spatial and temporal niche heterogeneity, anthropogenic pressure imposes a further stress on forest tree populations, often challenging their adaptive abilities (Mueller-Starck and Schubert, 2001). Hence, governments throughout the world are increasingly aware of the need for forest reproductive material that is genetically diverse and locally adapted (Geburek and Heinze, 1998; Kanowski, 2000). Unfortunately, the actual genes involved in adaptation to specific habitats or niches have very rarely been identified in trees (but see Frewen et al. (2000)), and, to our knowledge, molecular variation in such genes has never been tracked across the distribution range for any species. This unfortunate lack of information can be attributed to difficulties in identifying the relevant genes, as outlined below.

For many tree species, variation in ecologically important traits has been studied by provenance trials, that is, by comparing the performance of plants from different origins in homogeneous environments (Wright, 1976). Provenance trials, similar to reciprocal transplantation experiments, may be useful for testing the adaptive potential (or evolutionary constraints) of plant species in response to global warming, as demonstrated by a recent study on annual species (Etterson and Shaw, 2001). However, the power of provenance trials is rather limited if the goal is to identify the actual genes or quantitative trait loci (QTLs) involved in local adaptation. This is the case because in provenance trials, genotypes from geographically separated populations are mixed. Therefore, any association between traits and markers, potentially indicative of physical proximity along the chromosomes, will be confounded with the statistical interdependence caused by admixing alleles from different populations across all loci in the genome (Weir, 1996; Lynch and Walsh, 1998). Also, provenance trials may be too slow and time-consuming in forest trees, given the probable pace of climate change.

Many researchers have attempted to draw inferences about adaptive processes by population genetic surveys with markers that are either neutral, or whose adaptive significance is unknown. Indeed, indirect evidence suggests that sampling across ecological barriers to gene flow (e.g. across elevational gradients) can disrupt patterns of isolation by distance at neutral markers, thereby revealing past selection pressures and/or restricted gene flow (Williams and Arnold, 2001). However, it is unlikely that this approach will allow direct inferences about adaptively important genes, since recombination will quickly erase associations between the markers assayed and the genes responsible for fitness differences (Weir, 1996; Hedrick, 2000). In fact, most studies testing for concordance in patterns of variation at adaptive traits and neutral markers either report negative results (e.g. Savolainen and Hedrick, 1995; Karhu et al., 1995; McKay and Latta, 2002), or similarities due to the same historical events rather than selection (Lagercrantz and Ryman, 1990).

Maternally inherited chloroplast (cp) DNA markers yielded valuable information about genetic variability associated with local populations or provenances (e.g. Petit et al., 1997; Ferris et al., 1998). However, as pointed out by Kremer et al. (2002), the chloroplast genome is unlikely to contain genes sufficient to account for variation in complex adaptive characters. Indeed, in a large survey of oak stands (*Quercus robur* and *Q. petraea*) and provenance trials throughout Europe, no association was detected between chloroplastic divergence and phenotypic traits (Kremer et al., 2002).

An alternative method for identifying the molecular genetic basis of adaptive phenotypic variation involves the use of quantitative trait locus (QTL) mapping (Tanksley, 1993; Zeng,

1994; Kao et al., 1999). The QTL approach allows the identification of molecular markers that are physically linked to the genes of interest, and colocalization of QTLs and candidate gene sequences may lead to the identification of exactly those genes that account for variation in adaptive traits (Frewen et al., 2000; review by Mauricio, 2001).

Unfortunately, the application of the QTL methodology to forest trees poses several difficulties. First, standard QTL analysis (Tanksley, 1993; Mauricio, 2001) requires experimental crosses that are time-consuming and often impossible to obtain for trees (but see Hurme et al. (2000) for an interesting solution). Second, both QTL and candidate gene analysis require sufficient phenotypic variance and marker polymorphism in the "mapping population" (Karp et al., 1997; Lynch and Walsh, 1998), two requirements that reduce the choice of suitable crosses further. Third, identifying adaptive variation requires that the adaptive value of traits, QTLs, and candidate genes be assessed in the wild (Lexer et al., 2003c), that is, directly in the forest stand where fitness differences really matter. A logical conclusion from these difficulties would be to study QTLs in natural intraspecific populations, an approach that should theoretically be feasible in many animal or plant species (Luo et al., 2000; Wu and Zeng, 2001). However, such analyses may be difficult in forest trees, because linkage disequilibrium (LD) between markers and traits will decay quickly in large outbreeding plant populations (Weir, 1996; Hedrick, 2000).

In the present paper, we discuss alternative experimental designs based on natural hybrid zones. We outline the use of wild annual sunflowers (*Helianthus* spp.) as a model system for exploring the hybrid zone approach. More specifically, we summarize a series of studies involving transplanted experimental sunflower hybrids (Lexer et al., 2003a,b) and natural hybrid zones (Rieseberg et al., 1999a; Rieseberg and Buerkle, 2002). The main goal of these studies was to assess the role of hybridization in the origin of ecological divergence in annual sunflowers. We discuss the evolutionary implications of this work, as well as practical implications for comparable studies in forest trees, such as assays of the adaptive value of individual chromosome blocks or candidate genes directly in natural hybrid zones. We close by discussing the potential of the approach in selected forest tree genera.

## 2. Materials and methods

### 2.1. Study system Helianthus

The wild, annual sunflowers (*Helianthus* section *Helianthus*) include 12 self-incompatible, diploid ($n = 17$) species (Schilling and Heiser, 1981). The two most widespread species, *Helianthus annuus* and *H. petiolaris*, are abundant in the central and western US and have been the focus of our studies. They are easily distinguished by several morphological and chromosomal features (Heiser, 1947; Chandler et al., 1986), belong to divergent clades based on chloroplast DNA (Rieseberg et al., 1991) and nuclear ribosomal DNA variation (Rieseberg, 1991), and have different ecological requirements. In general, *H. annuus* is restricted to heavy, clay soils and *H. petiolaris* to dry, sandy soils. Nonetheless, these two habitats often are found in close proximity throughout the central and western US, resulting in the production of innumerable hybrid swarms or "mosaic" hybrid zones (Harrison and Rand, 1989). In addition, molecular data indicate that these two species gave rise to at least three diploid hybrid species in nature, *H. anomalus*, *H. deserticola*, and *H. paradoxus* (Rieseberg et al., 1990; Rieseberg, 1991). All three are adapted to extreme and novel habitats (sand dunes, desert floors, and salt marshes, respectively). It appears that the novel trait combinations required to colonize these extreme habitats originated by hybridization between the two parental species, *H. annuus* and *H. petiolaris*.

### 2.2. Plant materials

Two kinds of plant materials were used throughout this study: (i) experimental $BC_2$ hybrids between *H. annuus* and *H. petiolaris*, (ii) natural hybrids sampled in hybrid zones between the same two parental species. The $BC_2$ hybrids were used for transplantation experiments with the aim of studying selection on individual adaptive traits and QTLs. The natural hybrids were used for studying introgression of individual chromosome blocks across four replicate hybrid zones, and test for associations among markers and phenotypic traits.

**2.2.1. Experimental $BC_2$ hybrids—**Experimental $BC_2$ hybrids for the transplantation experiment were obtained by crossing two wild accessions of *H. annuus* and *H. petiolaris* and backcrossing a single interspecific $F_1$ to a second individual of *H. petiolaris*. Because of the near sterility of the $F_1$, only 38 $BC_1$ plants could be generated. These 38 plants were subjected to a second round of backcrossing toward a single, third individual of *H. petiolaris* to obtain the $BC_2$. Two-hundred-and-fifty-four $BC_2$ hybrids were transplanted into salt marsh habitat of the ancient hybrid species *H. paradoxus* at the seedling stage, as part of a selection experiment to study the role of hybridization in the origin of salt adaptation (Lexer et al., 2003a,b). For 172 of these plants, DNA could be extracted for molecular marker genotyping (Lexer et al., 2003b).

**2.2.2. Natural hybrids from sunflower hybrid zones—**The four natural hybrid zones (*H. annuus* × *H. petiolaris*) discussed here have been described in detail elsewhere (Rieseberg et al., 1999a; Buerkle and Rieseberg, 2002). Each zone is less than 50 m in width, occurs in human-disturbed sites, and is distributed along a habitat gradient. For each zone, 4–5 seeds were collected from 10 to 20 plants sampled along a transect from the center to the *H. annuus* edge of the zone, and seeds were propagated in the Indiana University greenhouses (total sample size $N = 228$). Also, reference populations of each parental species were sampled for comparative purposes ($N \sim 35$ per species).

### 2.3. Field and laboratory methods

**2.3.1. Experimental $BC_2$ hybrids—**The following characters were measured for the transplanted $BC_2$ hybrids: sodium content (Na), sulfur content (S), magnesium content (Mg), boron content (B), calcium content (Ca), potassium content (K), leaf shape (LFSHAP), and leaf succulence (LFSUC) as potentially adaptive traits, and survivorship in days as well as growth rate in the field as potential fitness proxies (described in detail in Lexer et al. (2003a)).

Total genomic DNA was isolated from ~100 mg of dried leaf tissue per plant using the DNeasy plant mini kit (QIAGEN, Valencia, CA), and microsatellites isolated from cultivated *H. annuus* (Tang et al., 2002) were used for genome-wide marker analysis. Microsatellite PCRs were performed in 96 well-format and analyzed on an ABI 3700 automated sequencer as described by Burke et al. (2002) and Lexer et al. (2003b). A total of 71 microsatellite loci were typed in the experimental $BC_2$.

#### 2.3.2. Natural hybrids

The following characters were measured for plants from natural hybrid zones: 10 morphological characters including leaf ratio (length/width), the length of flowering stems, the length of disk flowers, phyllary width/shape/pubescence, stem pubescence, leaf serration, as well as chaff pubescence and color. Also, pollen viability was assayed for 100 pollen grains per plant using enzymatic staining (described in detail in Rieseberg and Buerkle (2002)).

DNA for molecular analyses was extracted as described above for $BC_2$ hybrids. Random amplified polymorphic DNAs (RAPDs; Williams et al., 1990) were used for studying

introgression patterns in the four replicate hybrid zones. RAPD reactions were analyzed using 1.5% TBE agarose gels and ethidium bromide staining as described by Rieseberg et al. (1999a). In total, 88 RAPD bands were analyzed.

## 2.4. Data analysis

**2.4.1. Experimental $BC_2$ hybrids—**The transplantation experiment with $BC_2$ hybrids was used for assaying the strength of selection on candidate adaptive traits and on the underlying QTLs. Selection on candidate traits was calculated as directional selection (affecting the mean of phenotypic traits), stabilizing/disruptive selection (affecting trait variances), and correlational selection (affecting trait covariances), following the methods of Lande and Arnold (1983). Briefly, directional selection gradients were estimated as partial regression coefficients using multiple regression, directional selection differentials were estimated as the covariances between traits and fitness, stabilizing/disruptive selection differentials as the covariances between relative fitness and the variance of each trait, and correlational selection differentials as the covariances between relative fitness and the pairwise products of character deviations from the trait means, following Lande and Arnold (1983) and Lynch and Walsh (1998). All analyses were run on standardized trait values. This multiple regression-based approach is outlined in more detail in Lexer et al. (2003a). Potassium (K) uptake was excluded from these analyses because it violated basic assumptions of regression analysis. The four mineral uptake characters Na, B, Mg, and S were replaced by a principal component in selection analyses, because they were highly correlated based on Pearson's correlation coefficients.

QTL analyses in experimental hybrids employed a $BC_2$ model. Briefly, marker alleles segregating from *H. annuus* were scored in a *H. petiolaris* genetic background. The QTLs for five candidate adaptive traits (Na, Ca, S, Mg, and K content in leaves) and one fitness character (survivorship in the salt marsh, measured in days) were mapped on an interspecific linkage map constructed for the same $BC_2$ cross, using composite interval mapping (CIM; Zeng, 1993, 1994) as described in detail by Lexer et al. (2003b). Genome-wide threshold values for declaring the presence of QTLs were determined by 1000 permutations for each trait (Churchill and Doerge, 1994), and QTL magnitudes were expressed as the percent phenotypic variation explained (PVE) in the $BC_2$.

Selection coefficients ($s$) for individual QTLs were calculated using the nearest molecular marker for each QTL (the marker closest to the LR peak) as a surrogate and survivorship in days as a fitness measure. Selection estimates were obtained for heterozygous $BC_2$ genotypes carrying a marker allele derived from *H. annuus*, as described by Lexer et al. (2003b). Because the degree of dominance cannot be tested in a back-cross breeding design, gene action was assumed to be purely additive ($h = 0.5$).

**2.4.2. Natural hybrids—**The hybrid zone data were subjected to an analysis of marker introgression as described in detail in Rieseberg et al. (1999a). Briefly, maximum likelihood (ML) estimates of hybrid indices were obtained for each plant collected in the hybrid zone, making use of marker loci with diagnostic or frequency differences between the two parental species. These hybrid indices estimate the "hybridity" of each individual and vary from 0 to 1. Next, estimates of over-all marker introgression were obtained *for each individual* on the basis of hybrid indices, and deviations from expected introgression rates were then calculated *for each locus* using likelihood ratio tests. Associations between pollen viability and molecular markers were tested using permutation procedures, and fertility reduction scores at each locus were scaled for the effects of neighboring loci in the genome, as described in Rieseberg et al. (1999a). In order to explore the potential of the hybrid zone approach further, product-moment correlations were calculated as a measure of association between markers, and phenotypic traits

in natural hybrids were analyzed using descriptive statistics (described by Rieseberg and Buerkle (2002)).

## 3. Results

### 3.1. Transplanted $BC_2$ hybrids—adaptive value of individual chromosome blocks

**3.1.1. Selection on candidate adaptive traits—**By studying associations between candidate adaptive traits and fitness in experimental $BC_2$ sunflower hybrids transplanted into the wild we were able to detect significant directional selection on three phenotypic traits: (1) leaf succulence, (2) Ca uptake, and (3) a principal component including Na and other elemental uptake characters. Directional selection on these traits was calculated in the form of directional selection gradients (Fig. 1) and directional selection differentials (Table 1, directional selection). The relationships among candidate adaptive traits expressed in this extreme salt marsh habitat is depicted in Table 2. Trait correlations (lower triangular matrix) are particularly strong between Na, S, Mg, and B uptake, which is why these traits were summarized by a principal component. Significant pairwise correlations were also observed between Ca uptake or leaf succulence and other characters (Table 2), however, these traits were not included in a composite variable, because additional multivariate analyses suggested they were largely independent (Lexer et al., 2003a).

The direction of selection on phenotypic traits (Fig. 1) contains important information about their possible functional significance. Positive directional selection on leaf succulence (salt succulence) and Ca uptake from the field soil confirms an important role for these traits in salt stress response in wild sunflowers (Yeo, 1998;Hasegawa et al., 2000;Welch and Rieseberg, 2002), and negative directional selection on Na uptake (and correlated minerals) indicates that net exclusion of Na from leaves provides a fitness advantage in the salt marsh. Surprisingly, no significant selection was detected for leaf shape (leaf length/width), a diagnostic morphological character in sunflowers (Fig. 1; Table 1).

While no stabilizing or disruptive selection was detected, an interesting result was obtained regarding the change in trait correlations due to selection (Table 1, correlational selection). Only one pair of traits experienced a significant change in trait correlations after correction for multiple tests, namely Ca and Na uptake (Table 1). Also, Ca/Na ratios were under strong positive directional selection in this habitat ($s = +0.294$). This indicates an adaptive role for increased Ca uptake coupled with a greater capacity for Na exclusion in the salt marsh, a scenario that is well supported by the molecular salt tolerance literature (Hasegawa et al., 2000). In conclusion, although this experiment was conducted to study the origin of a wild sunflower hybrid species (see Section 4), our dataset also provides an example for the usefulness of phenotypic selection experiments in choosing candidate adaptive traits for QTL analyses.

**3.1.2. Selection coefficients for adaptive QTLs—**Assaying the transplanted $BC_2$ hybrids for 71 mapped microsatellite markers allowed us to conduct a genome-wide scan for quantitative trait loci (QTLs) expressed in extremely saline “hybrid” habitat. In total, 14 elemental uptake QTLs and 3 fitness QTLs, controlling survivorship in the salt marsh, were detected in the $BC_2$. The three survivorship QTLs cumulatively explained 38% of the temporal variation in survivorship in the wild, and the elemental uptake QTLs cumulatively explained between 21% (S uptake) and 78% (Ca uptake) of the variation in each elemental uptake trait (detailed presentation in Lexer et al. (2003b)). Notably, all three survivorship QTLs were closely correlated with QTLs for Ca and K uptake (Fig. 2A), Na and Mg uptake (Fig. 2B), or Na uptake (Fig. 2C). In all three cases, the one-LOD support intervals of elemental uptake and survivorship QTLs overlapped, and the microsatellite markers closest to the QTL likelihood

peaks were the same for both classes of QTLs (Fig. 2). Our results suggest that we have identified three chromosomal blocks associated with ecological selection in the wild.

The strength of natural selection for elemental uptake QTLs was examined by calculating selection coefficients for heterozygous $BC_2$ plants carrying molecular marker alleles derived from *H. annuus*. The selection coefficient was +0.126 for the tightly linked or pleiotropic QTLs controlling Ca and K uptake on linkage group 1 (Fig. 2A), −0.084 for the correlated QTLs controlling Mg and Na uptake on linkage group 4 (Fig. 2B), and −0.094 for the Na uptake QTL on linkage group 17b (Fig. 2C). The results indicate how the increased phenotypic variance and marker polymorphism present in a fairly complex hybrid pedigree can be used to map adaptively important QTLs directly in natural habitat. Although the present experiment was based on experimental hybrids, similar studies are conceivable for natural hybrid zones.

### 3.2. Introgression in natural hybrid zones

Studying introgression patterns of mapped molecular markers in replicate hybrid zones allowed the identification of chromosomal blocks that introgressed significantly more frequently or less frequently than expected, as exemplified by Fig. 3. Introgression patterns across replicate hybrid zones were largely concordant, thereby excluding genetic drift as being an important factor in generating the observed deviations from neutral expectations (presented in detail in Rieseberg et al. (1999b) and Buerkle and Rieseberg (2001)). For 26 chromosomal segments, introgression was significantly reduced, indicating that the genetic basis of the species' barrier is complex. Notably, many of the negatively selected chromosomal segments were from linkage groups that are known to be *collinear* between the two parental species (e.g. Fig. 3). These collinear chromosomal segments, equivalent to QTLs, likely contain one or more genes that contribute to reproductive isolation between these wild sunflower species. For chromosomal segments close to rearrangements, it is difficult to disentangle the effects of the chromosomal rearrangement from those of linked genes.

To determine the likely cause of the reduced rates of introgression for these chromosomal segments, we searched for correlations with an important reproductive isolating mechanism, pollen sterility. Significant associations were found with 16 of the 26 segments, providing a straightforward explanation of why this subset of chromosomal segments is negatively selected in hybrids (Rieseberg et al., 1999a). In an attempt to understand why the remaining 10 segments were negatively selected, we assayed all plants for numerous morphological differences between the species that we thought might contribute to the ecological divergence between them (Rieseberg and Buerkle, 2002). Unfortunately, the phenotypic data were too skewed toward one of the parental species, *H. annuus*, to allow meaningful analyses (Fig. 4). Although this was a disappointing result, the results did inform us regarding molecular marker requirements for hybrid zone QTL studies (Section 4.2.1) and sampling strategies for obtaining the ideal distribution of phenotypic variance in QTL analyses (Section 4.2.2).

## 4. Discussion

### 4.1. Selection on individual chromosome blocks in the wild—implications for hybrid speciation in sunflowers

The transplantation experiment presented here was designed to study the role of hybridization in the origin of novel adaptation in a wild diploid sunflower hybrid species, *Helianthus paradoxus*. By generating an experimental backcross population between the two parental species, *H. annuus* and *H. petiolaris*, and transplanting them into the extremely saline habitat of the natural hybrid species, we simulated what may have been the earliest steps in the speciation process that gave rise to *H. paradoxus* in nature. Our experiment allowed us to test the hypothesis that salinity adaptation in the hybrid neospecies resulted from the

complementary action of additive QTL alleles dispersed between the parental species. This process is thought to be responsible for the generation of extreme (transgressive) phenotypes in segregating hybrid populations (Tanksley, 1993; Rieseberg et al., 1999b). Transgressive segregation through complementary gene action provides a plausible and simple explanation for the rapid evolution of habitat or niche divergence often observed in plant hybrid lineages (Rieseberg et al., 1999b; Lexer et al., 2003b).

Our results from this transplantation experiment concord with the "complementary gene action" hypothesis. First, for all of the candidate adaptive traits (Welch and Rieseberg, 2002), one or more back-cross hybrids had phenotypes that exceeded the range of the parental species and the mean phenotype of the natural hybrid species, *H. paradoxus*. Second, the candidate adaptive traits were found to be under strong directional selection in the habitat of the natural hybrid species (Fig. 1, Table 1). Third, three chromosomal blocks were detected in this study that were associated with QTLs conferring differences in survivorship in the salt marsh, and all three of them also had a significant effect on elemental uptake from the soil (Fig. 2; Lexer et al., 2003b). The fitness effects of these genomic blocks derived from the donor parent in the $BC_2$ design, *H. annuus*, were in opposing directions (Fig. 2). QTL alleles with opposing effects were also detected for each of the elemental uptake characters (not shown; Lexer et al., 2003b). It is easy to see how these QTL alleles with opposing effects may be re-shuffled by recombination in early generation hybrids to generate a small number of transgressive genotypes with all QTL alleles in one direction, and this is exactly what is predicted by the "complementary gene action" hypothesis (Rieseberg et al., 1999b). It is therefore likely that selection for extreme (transgressive) genotypes in early hybrid generations did indeed facilitate the colonization of an extreme salt marsh habitat by *H. paradoxus* (Lexer et al., 2003a,b). More generally, our data provide support for the long-standing view that hybridization may provide the necessary genetic variation for natural selection to act upon (Anderson, 1949; Stebbins, 1959; Lewontin and Birch, 1966; Arnold, 1997; Barton, 2001).

### 4.2. Estimating the selective value of adaptive QTLs in hybrid zones—a possible approach for forest trees?

As outlined earlier in this paper, a major impediment to identify adaptive genetic variation in forest trees is the difficulty of estimating the adaptive value of QTLs or candidate genes in "conventional" crosses. Transplantation experiments like the one presented here for sunflowers are not feasible in forest trees, if the aim is to measure adaptive traits and fitness characters on *adult* plants. Association studies in natural hybrid zones may help to circumvent this problem, since genetic mapping of QTLs *and* selection assays can be combined directly in natural populations. Also, natural hybrid zones contain increased phenotypic variance and marker polymorphism that can be exploited for genetic analyses (Barton and Hewitt, 1985; Harrison, 1990; Barton and Gale, 1993). In particular, many of the traits or QTLs that are most important to adaptation may be invariant *within* populations or species (Orr, 2001), while the same traits (QTLs) are often variable in hybrids (reviews by Rieseberg et al. (2002) and Lexer et al. (2003c)). An important notion is that the two approaches presented here, selection experiments at the QTL level and introgression studies in hybrid zones, can potentially be combined. This may allow an assessment of the adaptive value of individual QTLs in natural forest stands, and our data on sunflower hybrid zones may inform such studies.

**4.2.1. Marker requirements—**A graphical result that emerges from our hybrid zone data in sunflowers is that the number of *informative* markers must be large enough to allow for genome-wide introgression studies. Analyzing introgression patterns as in Fig. 3 requires diagnostic markers, or markers with allele frequency differences between the two parental taxa (Rieseberg et al., 1998;1999a). Note that the potential for identifying sufficient numbers of markers not only depends on the degree of genetic differentiation between the hybridizing

populations, e.g. rather high differentiation in sunflowers (Rieseberg et al., 1999a), intermediate differentiation in North American *Populus* species (Martinsen et al., 2001), rather low differentiation in European oaks (Bodénès et al., 1997), but also on the molecular marker systems employed. Large-scale sequencing of expressed sequences (ESTs) in forest trees (http://dendrome.ucdavis.edu) as well as development of Bayesian approaches to hybrid identification (Anderson and Thompson, 2002) hold the promise to extend the applicability of this approach further in the future.

Fortunately, results from human populations (Stephens et al., 1994) indicate that in order to be informative, alternative allelic forms need not be close to fixation in the pure parental populations, as was the case in the present sunflower study. Rather, loci with smaller allele frequency differences between the parental populations (=0.3) should also produce sufficient linkage disequilibrium (LD) for association studies when admixed in hybrid populations. This may allow the detection of marker-trait associations in species pairs with little genetic differentiation, as suggested by preliminary results in European oaks (Saintagne et al., 2004).

**4.2.2. Sampling strategies—**As shown in Fig. 4, phenotypic distributions in the hybrid zone dataset were highly skewed towards *H. annuus* like phenotypes. This is not surprising, since these samples were collected to estimate introgression from *Helianthus petiolaris* into *H. annuus*, and not primarily for QTL analyses. However, the phenotypic distributions in Fig. 4 also illustrate an important consideration in sampling hybrid zones in trees.

In many forest taxa, introgression has been shown to occur preferentially in one direction (e.g. Bacilieri et al., 1996; Keim et al., 1989). Therefore, the hybrid individuals that are most informative for QTL analyses from a genotypic perspective may not be the most informative ones with respect to their phenotypes. This is because, from a genotypic perspective, the ideal dataset for QTL mapping consists of 5th to 12th generation hybrids. In these plants, recombination should have had sufficient time to break up the parental species' genomes (Briscoe et al., 1994; Rieseberg and Buerkle, 2002). However, with respect to phenotype, the most informative hybrids will have a hybrid index near 0.5 (Fig. 4) and will therefore be early generation hybrids or even $F_1$'s.

An optimal sampling strategy to resolve this paradox might involve screening a large number of hybrids with a moderate number of nuclear markers (20–30 loci) in order to identify the most recombinant genotypes (later generation hybrids). In these plants, linkage disequilibrium (LD) between loosely linked genes should still be detectable, while LD between unlinked genes should have decayed by then (Briscoe et al., 1994). Including only plants that are highly recombinant, and that contain genetic material from both parental species in a balanced distribution, will provide the basis for sampling a broad phenotypic distribution as outlined in Fig. 4. This may require sampling several hundred hybrids in the initial screening step, while sample sizes for the genome-wide QTL analysis will depend on the actual size of genome blocks in hybrids (below). Plants that were screened with markers but not included in the QTL analysis could be of interest elsewhere, e.g. for analyzing hybrid zone structure and inferring management and conservation measures, or simply for identifying and maintaining interesting genotypes for breeding and selection programs.

**4.2.3. Estimating selection in natural hybrid zones—**Perhaps the single most important difference between our selection study with transplanted experimental hybrids (Section 3.1) and selection assays in natural hybrid zones is the fact that hybrid zones contain substantial environmental variability that may confound marker-trait associations, particularly for adaptive traits strongly affected by environmental variance. In our transplantation experiment, environmental heterogeneity was taken into account by dividing the field site into "blocks", and including them as random factors in ANOVA models (Lexer et al., 2003a).

However, this procedure may not be applicable to hybrid populations in forest trees because of the increased environmental heterogeneity found in natural forest stands, and because of the size of the geographical areas studied. A more fruitful approach—other than the establishment of common garden trials—may be to record key habitat factors for every tree sampled, and include them as covariates in selection models (Sokal and Rohlf, 1995).

A second major difference between selection assays in hybrid zones and transplantation studies with experimental hybrids is that a priori pedigree information is generally not available for hybrid zones. However, this is less likely to be a problem, because diagnostic molecular markers will reveal the exact species origin of each chromosomal segment (e.g. Rieseberg et al., 1999a; Martinsen et al., 2001; Rieseberg and Buerkle, 2002). Therefore, fitness differences at codominant markers can be assigned to QTL alleles derived from each parental species. This will yield reliable estimates of the strength of selection, as long as a major proportion of the phenotypic variation in the trait of interest can be attributed to interspecific differences (Orr, 2001). In addition, the use of highly polymorphic codominant markers holds the promise to reconstruct even complex pedigree structures in wild populations (Queller and Goodnight, 1989; Blouin et al., 1996; Lynch and Ritland, 1999), potentially reducing the problem to an analysis of hybrid crosses between individual trees.

### 4.3. Potential of the hybrid zone approach in selected forest tree genera

**4.3.1. Populus—**The genus *Populus* (poplars, cottonwoods, and aspens) is certainly the most advanced genetic model species in forest trees, with its relatively small genome size (550 Mb; $2n = 38$), and its complete genomic sequence becoming available soon (Bradshaw et al., 2001; Wullschleger et al., 2002). Species barriers in *Populus* are known to be porous, and hybridization is frequent wherever two related species have overlapping ranges (Keim et al., 1989; Rajora and Dancik, 1992; Eckenwalder, 1996; Martinsen et al., 2001).

One aspect renders *Populus* particularly suitable for QTL studies in natural hybrid zones: species barriers appear to be *genic* rather than chromosomal. This is expected because interspecific hybrids in *Populus* are generally diploid (Eckenwalder, 1996), and homologies between different species' linkage maps are widespread (Cervera et al., 2001). Hence, species barriers appear to be composed primarily of pairs of complementary genes conferring intrinsic incompatibilities (reduced hybrid fertility) or genes involved in habitat or niche divergence, i.e. exactly those groups of genes that are so important to forest ecology and management.

Molecular gene introgression studies have been conducted for hybrid zones among North American cottonwoods (*Populus fremontii* × *P. angustifolia*; Keim et al., 1989; Martinsen et al., 2001). The results suggest that different genes or chromosomal blocks do introgress at different rates, or vary in their potential to spread within the recipient species' populations (Martinsen et al., 2001). In addition, the increased genetic variance present in hybrids *P. fremontii* × *P. angustifolia* has been utilized for examining interactions with herbivores and pathogens, and some of the best evidence for the importance of hybrid zones for biodiversity in the plant kingdom have been conducted in these species (Whitham, 1989; Whitham et al., 1999).

In other hybrid systems, such as *P. alba* × *P. tremula* (Rajora and Dancik, 1992), divergent habitat preferences related to flooding or other disturbance regimes along river floodplains (Karrenberg et al., 2002) may provide a venue for introgression of adaptive traits. Hybrid zones between *P. alba* and *P. tremula* occur along several major European river systems (Rajora and Dancik, 1992), thus providing a "replicated natural hybridization experiment" for comparing QTLs as well as fitness effects of traits, QTLs, and candidate genes along parallel evolutionary trajectories.

With respect to possible applications in forestry, it is important to note that in poplar breeding, adaptive traits like abiotic stress tolerance or disease resistance can often only be obtained from other species (Stettler et al., 1996). Insights into the function of adaptively important genes in this genus may thus have direct implications for the breeding of new cultivars, either by 'gene-assisted' traditional crossings (i.e. supported by molecular analyses of offspring for suitable recombinants), or by transformation-based gene transfer between closely related genomes (Bradshaw et al., 2001; Wullschleger et al., 2002).

**4.3.2. Fraxinus—***Fraxinus* (ash) is another diploid genus ($2n = 46$) that may be useful as a model for hybrid zone studies among temperate forest trees. *Fraxinus* is represented by *F. excelsior*, the common ash, throughout most of central Europe. The Mediterranean *F. angustifolia*, narrowleaf ash, extends into the Pannonian basin (Hungary and neighboring regions), but has only recently been recognized as a true species—both are very similar morphologically (Jelem, 1974; Fukarek, 1971). *Fraxinus angustifolia* is a floodplain species, with considerable tolerance to prolonged flooding. It appears that through introgression of *F. angustifolia* alleles, flooding tolerance finds its way into the *F. excelsior* genome, enabling this upland species to colonize frequently flooded sites more effectively further upstream where *F. angustifolia* meets its lower temperature limit (Volk, 2002; Jelem, 1974).

Little is known about the genetic make-up of hybrid populations of *F. excelsior* × *F. angustifolia*, e.g. about the frequency of different hybrid generations or genotypic classes. However, since both species reached their present distribution soon after the retreat of the glacial ice sheets approximately 100–200 tree generations ago (Huntley and Birks, 1983; Tinner and Lotter, 2001), later generation hybrids should be frequent. Although the hybrid zones of *F. excelsior* × *F. angustifolia* are considerably older than those studied in wild sunflowers, the number of generations of hybridization is roughly equivalent. This may provide ideal conditions for QTL mapping, since a large number of recombination events should have accumulated in these hybrids.

Unfortunately, *Fraxinus* species are comparatively poorly characterized genetically. Although a moderate number of molecular markers have been developed for ash (e.g. Jeandroz et al., 1996; Morand-Prieur et al., 2002), no genetic linkage map is available. However, development of such tools might benefit from research on another member of the Oleaceae, the cultivated olive *Olea europaea* (e.g. Rallo et al., 2000; Sefc et al., 2000; De La Rosa et al., 2002). The development of genomic tools in ash and olive, in combination with the fact that *Fraxinus* spp. and their hybrids are still comparatively easy to classify in the field (compared to, say, willows), all make ash a highly interesting candidate for hybrid zone-based QTL mapping approaches.

**4.3.3. Quercus—**Oak (*Quercus*) is a particularly complex genus (Burger, 1975; van Valen, 1976), and its porous species boundaries and numerous intermediate forms attracted the curiosity of early evolutionists (Darwin, 1859). Clearly, hybridization and introgression among diploid ($2n = 24$) species have played an important role during oak evolution (Petit et al., 1997; Belahbib et al., 2001; Howard et al., 1997). Despite relatively high levels of interspecific gene exchange (Whittemore and Schaal, 1991; Petit et al., 1997; Belahbib et al., 2001), divergent ecological selection has generated substantial interspecific differentiation in adaptive traits. This ecological divergence appears to account for the coexistence of closely related sympatric species within extensive hybrid zones, e.g. in the European white oaks *Q. robur* and *Q. petraea* (Kremer et al., 1993). These hybrid populations may be suitable for QTL mapping approaches as outlined in this paper, although the setting provided by these two species may be more difficult than those for other genera.

Extensive molecular marker surveys in *Q. robur* and *Q. petraea* indicate that only a small proportion of markers differ in allele frequencies between the species (Bodénès et al., 1997).

Analysis of the genomic locations of these markers suggests that only a limited number of genomic regions or “hot spots”, equivalent to QTLs, may separate two genomes that are otherwise poorly protected from interspecific gene flow (Saintagne et al., 2004). Although the porosity of oak genomes may not permit genome-wide QTL scans in oak hybrid zones, the important adaptive traits differentiating oak species are likely to be controlled by the segments differentiating their genomes. So only some additional fine-mapping may be required. Of course, it will be interesting to see if candidate genes involved in divergent soil preferences are located within genomic regions that differ between the two species. It also should be noted that diagnostic differences are not required for admixture linkage disequilibrium mapping (Stephens et al., 1994), so the approaches outlined in the present paper may be more applicable to oaks than is superficially apparent.

### 4.4. Conclusions

Natural hybrid zones are promising tools for identifying adaptive genetic variation in organisms that are long-lived or otherwise of limited genetic tractability. They potentially allow forest geneticists to circumvent the need for experimental multi-generation crosses, offer increased marker polymorphism and phenotypic variance in adaptive traits, and hold the promise of genetically mapping QTLs or candidate genes for phenotypic traits *and* simultaneously assessing their adaptive value directly in natural forest stands.

In the present paper, we attempted to draw first conclusions about the applicability of this approach using existing data from an annual plant genus: wild annual sunflowers (*Helianthus* spp.). By combining QTL mapping and selection assays on transplanted sunflower hybrids, we were able to assess the adaptive value of individual chromosome blocks in the habitat of a wild sunflower hybrid species. Our results indicate that QTL alleles from both parental species are required to provide a selective advantage in the wild, thereby indicating an important role for hybridization in the rapid evolution of novel adaptation in this study system. Additional data from natural hybrids indicate that similar studies are possible directly in natural hybrid zones, provided that sufficient numbers of diagnostic markers are available, and that large enough number of recombinant hybrid genotypes can be identified.

The approaches outlined in this paper may be directly applicable to hybrid zones in forest trees, as outlined by a short literature review of three forest tree genera: *Populus* (poplars, aspens, cottonwoods), *Fraxinus* (ash), and *Quercus* (oak). Temperate tree genera are particularly amenable to this kind of study, because molecular markers and genetic linkage maps are available, as are extensive molecular and fossil datasets on their postglacial history (e.g. Huntley and Birks, 1983; Petit et al., 1997; Kremer et al., 2002). This allows forest geneticists to date the approximate age of hybrid zones, thereby providing an upper bound for the number of hybrid generations present. Such estimates will be extremely valuable in designing QTL experiments, since the age of individual hybrid crosses will dictate the size and frequency of parental genome blocks in hybrids (Martinsen et al., 2001) and the marker densities required.

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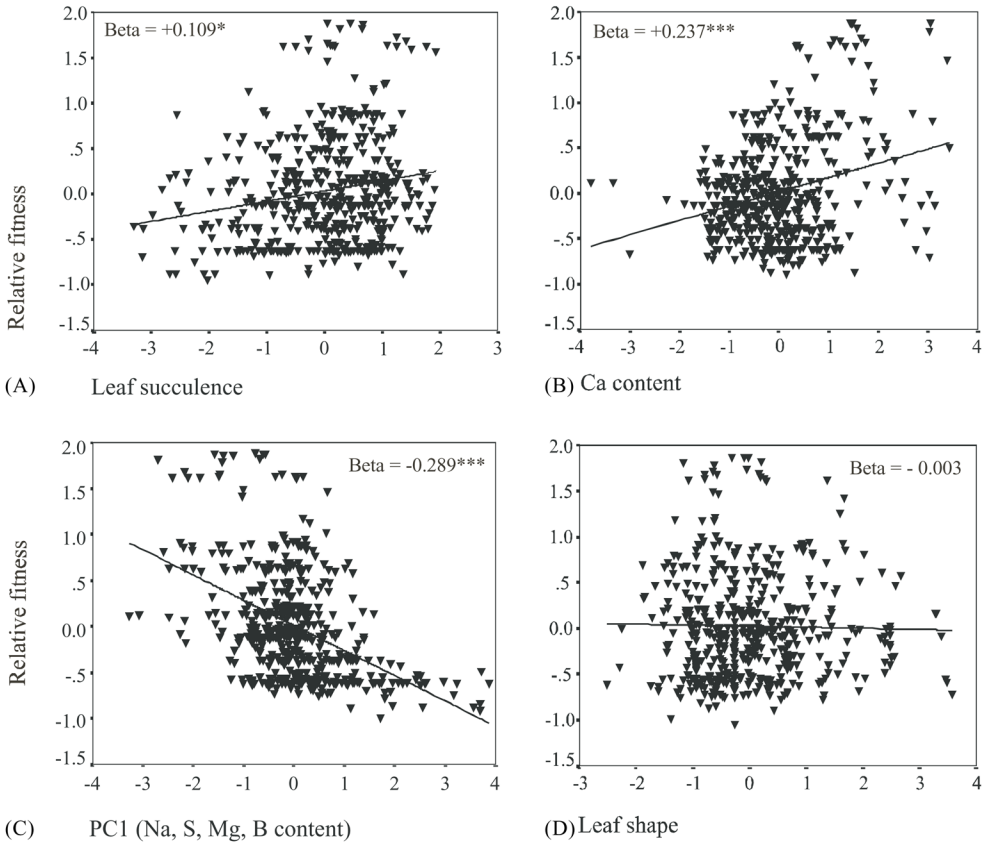


**Fig. 1.**

Partial regression plot of directional selection gradients on four candidate adaptive traits: (A) leaf succulence; (B) Ca content; (C) the first principal component (PC1) of Na, S, Mg, and B content; (D) leafshape. For each plot, the respective candidate trait and relative fitness (survivorship in days) were regressed on all other traits to obtain residuals from each regression. The relative fitness residuals were then regressed on the candidate adaptive trait residuals. The selection gradients (beta) given in the upper left or right corner of each graph correspond to regression coefficients estimated from the full regression model (*: $P < 0.05$; ***: $P < 0.005$). Residuals of candidate adaptive traits were measured in standard deviation units. The entire multiple regression model, including all four traits as well as blocks to account for environmental variation, accounted for 38% of the fitness variation in the $BC_2$.

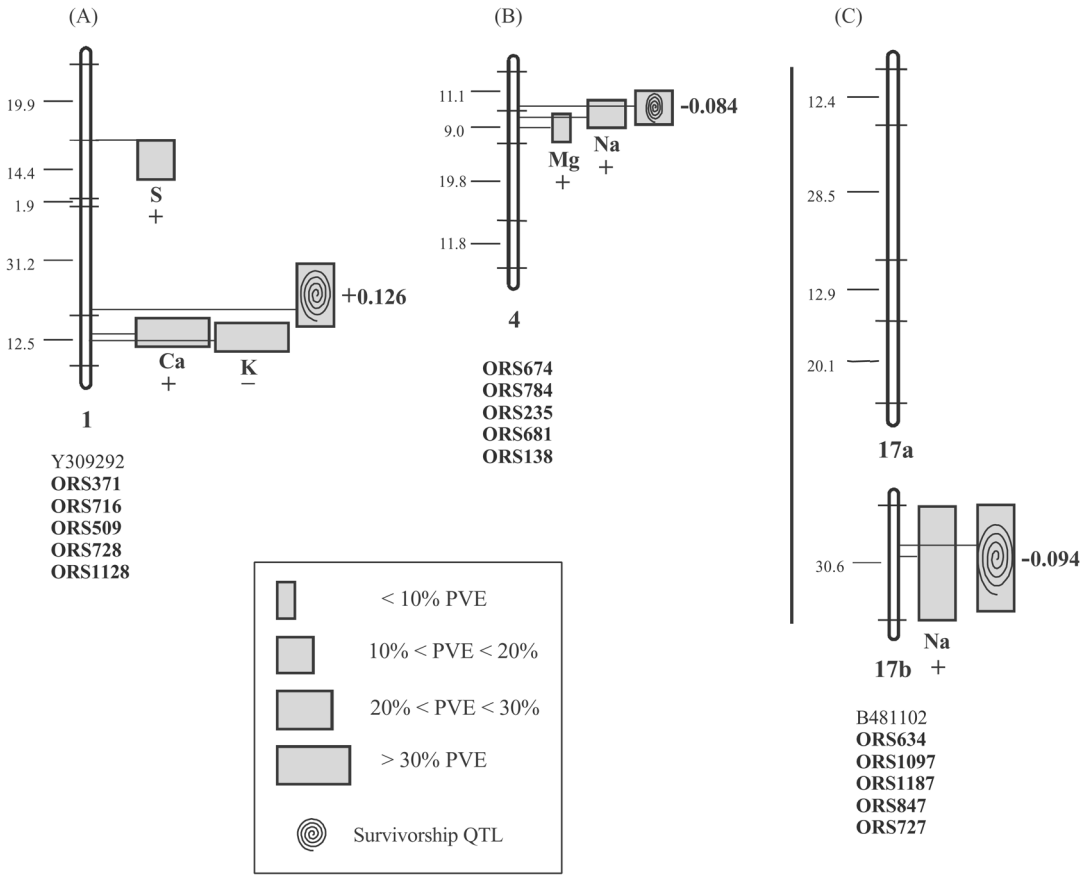


**Fig. 2.**

Selected linkage groups of a genetic map derived from a second generation backcross population ($BC_2$) of *H. annuus* × *H. petiolaris*, and QTL positions for survivorship and elemental uptake traits measured in the wild. Marker positions are shown by horizontal lines, and map distances between markers by numerals to the left of each group. Linkage groups were assigned according to microsatellite linkage maps for *H. annuus* (Burke et al., 2002; Tang et al., 2002). Marker groupings that differed between the intra- and interspecific maps (presumably due to pseudolinkage and/or fragmentation in the interspecific $BC_2$) are indicated by thick black lines at the left of the groups. QTL positions with one-LOD support intervals, additive effects (±), and QTL magnitudes are indicated by vertical bars to the right of each group. Marker names are listed according to order below each group. Marker names starting with ORS refer to microsatellites isolated from *H. annuus* (Tang et al., 2002). Two additional AFLP markers of the original linkage map were not assayed in the present field study. Selection coefficients for individual chromosome blocks are indicated to the right of each survivorship QTL. Redrawn from Lexer et al. (2003b).

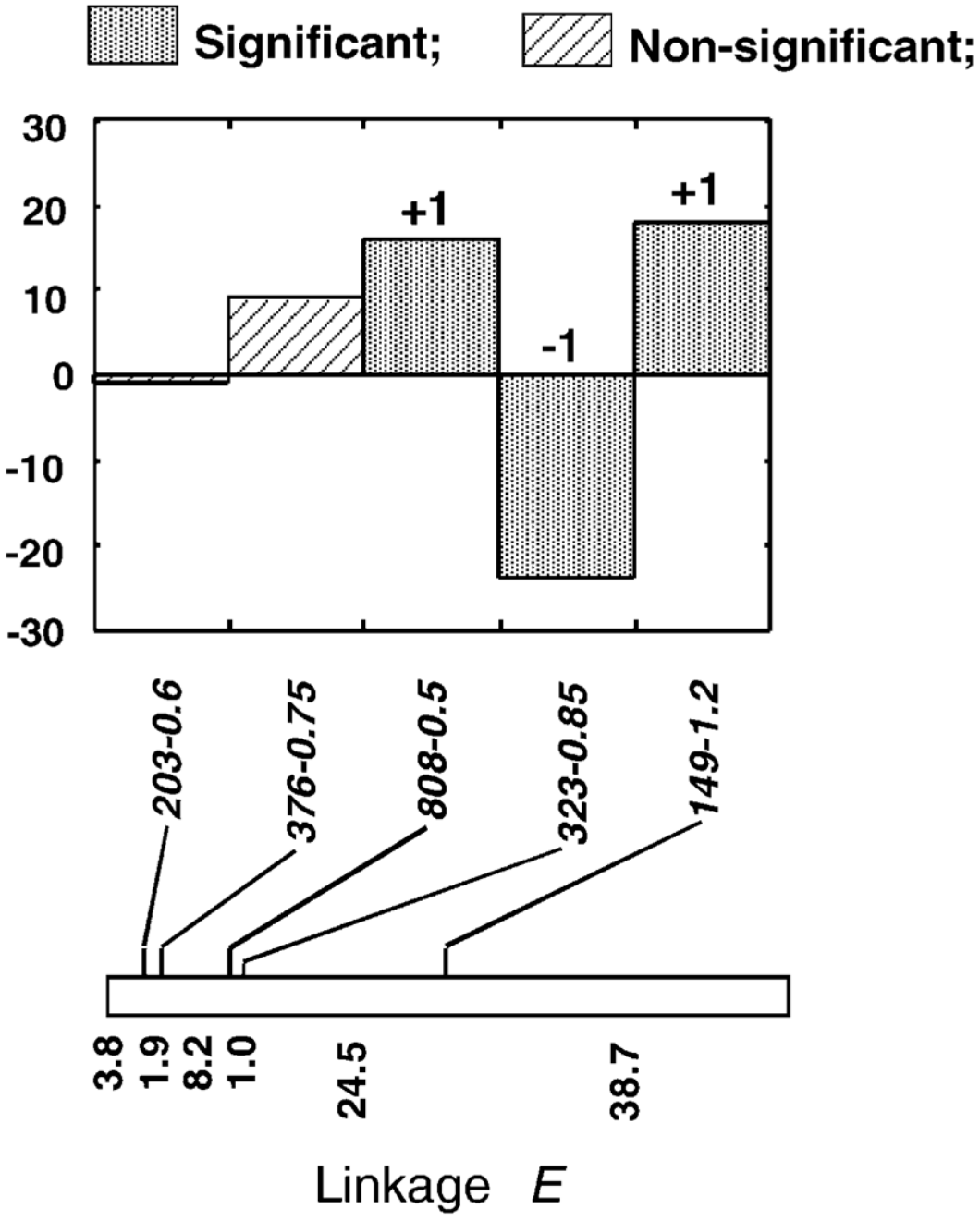


**Fig. 3.**

Direct count deviations from the expected number of introgressed marker alleles in three natural hybrid zones between *H. annuus* and *H. petiolaris*. The linkage group shown is collinear between the genomes of *H. annuus* and *H. petiolaris*. Mapped molecular markers are given above and map distances below the linkage group. Independently selected chromosome blocks are indicated by +1 or −1, depending on the direction of selection. Redrawn from Rieseberg et al. (1999b).

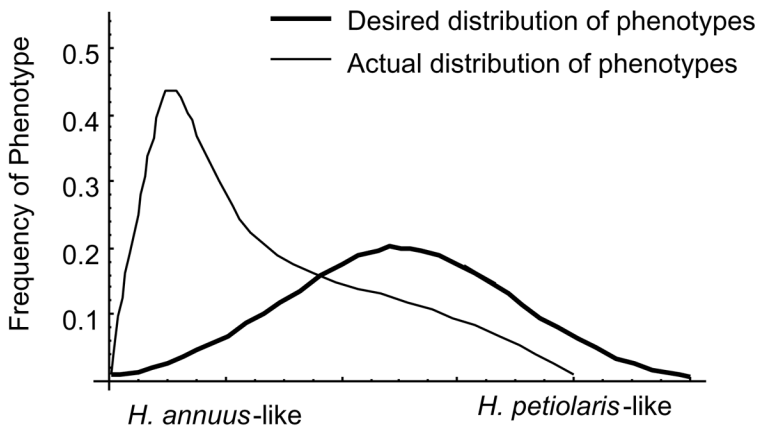


**Fig. 4.**

Observed vs. desired distributions of phenotypes for QTL mapping in wild sunflower hybrid zones. Redrawn from Rieseberg and Buerkle (2002).

**Table 1**

Directional and correlational selection differentials in transplanted $BC_2$ sunflower hybrids

| | Na | S | Mg | B | Ca | LFSHAP | LFSUC |
|---|---|---|---|---|---|---|---|
| Directional selection[a] | −0.250*** | −0.250*** | −0.250*** | −0.250*** | +0.062 | −0.003 | +0.132*** |
| Correlational selection[b] | | | | | | | |
| Na | | | | | | | |
| S | +0.058** | | | | | | |
| Mg | +0.079 | −0.009 | | | | | |
| B | −0.032 | −0.081 | −0.050 | | | | |
| Ca | **−0.229**** | −0.157** | −0.224*** | −0.163*** | | | |
| LFSHAP | −0.064 | −0.037 | +0.001 | −0.020 | +0.004 | | |
| LFSUC | +0.009 | +0.020 | +0.117 | +0.047 | +0.123** | +0.077 | |

[a] Directional selection differentials: significance levels for individual tests,

*** $P < 0.005$. Directional selection differentials for Na, S, Mg, and B are for the first principal component of these variables.

[b] Correlational selection differentials:

** : $P < 0.01$,

*** $P < 0.005$ for individual tests. Bold type: $P < 0.05$ after sequential Bonferroni correction. For trait abbreviations see text.

**Table 2**

Correlations and covariances between phenotypic traits in transplanted $BC_2$ sunflower hybrids

| | Na | S | Mg | B | Ca | LFSHAP | LFSUC |
|---|---|---|---|---|---|---|---|
| Na | | +0.492 | +0.452 | +82.560 | +0.300 | −0.113 | −1.522 |
| S | +**0.607**$^{***}$ | | +0.045 | +9.612 | +0.087 | −0.020 | −0.101 |
| Mg | +**0.675**$^{***}$ | +**0.441**$^{***}$ | | +12.938 | +0.096 | +0.017 | −0.135 |
| B | +**0.397**$^{***}$ | +**0.301**$^{***}$ | +**0.490**$^{***}$ | | +25.405 | +3.269 | −41.734 |
| Ca | +0.165$^{*}$ | +**0.312**$^{***}$ | +**0.415**$^{***}$ | +0.**354**$^{***}$ | | +0.111 | −0.244 |
| LFSHAP | −0.070 | −0.074 | +0.085 | +0.059 | +0.193$^{*}$ | | −0.001 |
| LFSUC | −**0.362**$^{***}$ | −0.145 | −**0.257**$^{**}$ | −0.**291**$^{***}$ | −0.163$^{*}$ | −0.001 | |

Upper triangular matrix: covariances. Lower triangular matrix: Pearson's correlation coefficients.

$^{*}$: $P < 0.05$,

$^{**}$: $P < 0{:}01$,

$^{***}$ $P < 0{:}005$ for individual tests. Bold type: $P < 0{:}05$ after sequential Bonferroni correction. For trait abbreviations see text.