

Natural selection for salt tolerance quantitative trait loci (QTLs) in wild sunflower hybrids: Implications for the origin of *Helianthus paradoxus*, a diploid hybrid species

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Abstract

For a new diploid or homoploid hybrid species to become established, it must diverge ecologically from parental genotypes. Otherwise the hybrid neospecies will be overcome by gene flow or competition. We initiated a series of experiments designed to understand how the homoploid hybrid species, *Helianthus paradoxus*, was able to colonize salt marsh habitats, when both of its parental species (*H. annuus* × *H. petiolaris*) are salt sensitive. Here, we report on the results of a quantitative trait locus (QTL) analysis of mineral ion uptake traits and survivorship in 172 BC₂ hybrids between *H. annuus* and *H. petiolaris* that were planted in *H. paradoxus* salt marsh habitat in New Mexico. A total of 14 QTLs were detected for mineral ion uptake traits and three for survivorship. Several mineral ion QTLs mapped to the same position as the survivorship QTLs, confirming previous studies, which indicated that salt tolerance in *Helianthus* is achieved through increased Ca uptake, coupled with greater exclusion of Na and related mineral ions. Of greater general significance was the observation that QTLs with effects in opposing directions were found for survivorship and for all mineral ion uptake traits with more than one detected QTL. This genetic architecture provides an ideal substrate for rapid ecological divergence in hybrid neospecies and offers a simple explanation for the colonization of salt marsh habitats by *H. paradoxus*. Finally, selection coefficients of +0.126, −0.084 and −0.094 for the three survivorship QTLs, respectively, are sufficiently large to account for establishment of new, homoploid hybrid species.

Keywords: ecological divergence, *Helianthus*, hybrid speciation, hybridization, natural selection, QTL

Received 1 October 2002; revision received 6 January 2003; accepted 6 January 2003

Introduction

A major goal in evolutionary biology is to estimate the fitness effects of individual mutations in natural populations. Such estimates are critical to a diversity of issues, ranging from the maintenance of genetic variation (e.g. Wayne & Mackay 1998), to the plausibility of Wright's shifting balance theory (Wright 1931), to the likelihood of sympatric or parapatric speciation (Maynard Smith 1966). Unfortunately, the strength of selection on individual mutations is difficult to measure. In many instances, for example, the gene causing variation in fitness has not yet been identified. Even if the correct gene has been determined, however,

it may not be possible to assign changes in fitness to individual mutations because of linkage disequilibria among multiple, linked mutations. Furthermore, estimates of lifetime fitness in nature are difficult to obtain in many organisms, particularly if they are long-lived or highly mobile (Endler 1986).

The situation is even more discouraging for the analysis of genes that contribute to species' differences. First, because the allelic polymorphisms of interest occur between species rather than within populations, linkage disequilibria may extend across the entire genome, vs. 0–800 kb for comparisons within outcrossing species (e.g. Dawson *et al.* 2002). Second, alleles that differentiate species are more likely to vary by multiple substitutions than those polymorphic within species. Third, it is likely to be difficult to recreate the ancestral habitat or genetic

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background in which a mutation originally arose and became fixed. As a result, the identification and ordering of the mutational steps that cause speciation represents a formidable challenge.

These difficulties are substantially reduced, however, for diploid or 'homoploid' hybrid speciation. In this mode of speciation, hybridization between genetically differentiated species gives rise to a stable, fertile and reproductively isolated hybrid lineage, but without a change in chromosome number (Rieseberg 1997). Reproductive isolation is largely achieved through the sorting of pre-existing genetic material, whether it be chromosomal rearrangements that differentiate the parental species ('recombinational speciation', Grant 1981; see also McCarthy *et al.* 1995; Rieseberg *et al.* 1995) or parental species' alleles that in combination allow the hybrid lineage to colonize a new ecological niche (Rieseberg *et al.* 1999; Buerkle *et al.* 2000; Lexer *et al.* 2003). Because new mutations are seemingly not required for homoploid hybrid speciation, speciation appears to occur rapidly, often in fewer than 50 generations (McCarthy *et al.* 1995; Ungerer *et al.* 1998; Buerkle *et al.* 2000). As a consequence, selection acts on phenotypic variation generated by shuffling fairly large chromosomal segments (Ungerer *et al.* 1998), rather than individual mutations or alleles. Chromosomal segments of suitable size for selection studies are easily generated in experimental hybrids.

Reconstruction of the ancestral habitat and genetic background for homoploid hybrid species is also straightforward. Computer simulations indicate that this mode of speciation is unlikely to be successful unless ecological divergence takes place very early in the speciation process (Buerkle *et al.* 2000). Habitats currently occupied by homoploid hybrid species are therefore likely to be similar to those in which the hybrid species originally arose. Finally, the ancestral genetic background may be reconstructed by hybridizing the parental species, if they still exist. Thus, we can come closer to replicating this form of speciation than perhaps any other, with the exception of allopolyploid speciation.

This study represents the latest in a series of experimental investigations of the origin of homoploid hybrid species in the annual sunflowers of the genus *Helianthus* (Rieseberg *et al.* 1996; Rieseberg 2000; Lexer *et al.* 2003). The annual sunflowers are particularly suitable for these kinds of studies, because 3 of the 11 species in this group (*H. anomalus*, *H. deserticola* and *H. paradoxus*) are stabilized homoploid hybrid derivatives of two widespread sunflowers, *H. annuus* and *H. petiolaris* (Rieseberg *et al.* 1990; Rieseberg 1991). Hopefully, studies of this replicated natural hybridization experiment will permit generalizations about the factors that facilitate or constrain this mode of speciation, and perhaps lead to a firmer understanding of speciation in general.

Initial experimental studies in this group focused on the role of fertility selection in shaping the genomes of both experimental and ancient hybrid lineages (Rieseberg *et al.* 1996) and the evolution of reproductive isolation as a by-product (Rieseberg 2000). More recently, attention has shifted to the evolution of the phenotypic changes responsible for ecological divergence in the three homoploid hybrid species (Schwarzbach *et al.* 2001; Rosenthal *et al.* 2002; Welch & Rieseberg 2002): *H. anomalus* is found in sand dune habitats, *H. deserticola* on the desert floor and *H. paradoxus* in brackish, saline marshes (Heiser *et al.* 1969; Rogers *et al.* 1982). All three habitats differ substantially from that of the parental species: *H. annuus* (mesic, clay-based soils) and *H. petiolaris* (drier, sandy soils). It appears that the extreme phenotypes exhibited by the hybrid species likely arose via hybridization and represent adaptations to the habitats in which these species occur (e.g. Lexer *et al.* 2003), but this has not been shown for all taxa or all traits.

Here, we focus on the origin of ecological divergence in *H. paradoxus*. Growth chamber experiments indicated that *H. paradoxus* is 5–14 times more salt tolerant than its parental species and pointed to greater leaf succulence and leaf sodium sequestration as possible mechanisms for increased tolerance (Welch & Rieseberg 2002). Field experiments, in which segregating second generation backcross (BC₂) plants, *H. annuus* (parent), *H. petiolaris* (parent) and *H. paradoxus* were transplanted into the natural saline salt marsh habitat of the latter, confirmed the greater salt tolerance of *H. paradoxus* and the role of leaf succulence as a contributing mechanism; positive directional selection was detected for leaf succulence (Lexer *et al.* 2003). The relationship between fitness and mineral ion uptake/sequestration was more complex, however, than suggested from the growth chamber experiments. The natural habitat of *H. paradoxus* is characterized by a complex mixture of mineral ions, including boron (B), calcium (Ca), magnesium (Mg), sulfur (S) and sodium (Na). Strong and positive directional selection was detected for Ca uptake, whereas strong negative selection was associated with the uptake of Na and related ions (Lexer *et al.* 2003). In addition, trait correlations decreased between Ca and Na uptake during the course of the experiment, suggesting that increased Ca uptake, coupled with greater exclusion of Na and related mineral ions, contributes to increased salt tolerance in these *Helianthus* species (Lexer *et al.* 2003). Finally, the ranges of trait values in the BC₂ were larger than those of the parentals for all traits, and at least one BC₂ plant exceeded the median *H. paradoxus* phenotype for each trait. Thus, it should be feasible for natural selection to assemble the *H. paradoxus* phenotype from an ancestral hybrid population in just a few generations.

This study extends the field experiment described above, by assaying the BC₂ population for molecular markers and then searching for correlations between fitness and segregating salt tolerance quantitative trait loci (QTLs). We

ask four specific questions related to the hybrid origin of *H. paradoxus*:

- 1 What is the genetic basis for the wide range of phenotypic trait values in the hybrid mapping population?
- 2 Are adaptive QTLs derived from both parental species as predicted by models of homoploid hybrid speciation?
- 3 Do QTLs for Ca uptake map to the same position as other mineral ions and, if so, how do the observed changes in genetic correlations come about?
- 4 What are the selection coefficients for individual QTLs and what do these estimates tell us about the likelihood of homoploid hybrid speciation?

Materials and methods

Plant materials

An interspecific BC₂ population between *Helianthus annuus* and *H. petiolaris* was used to identify QTLs and estimate QTL fitness; a BC₁ mapping design may have been preferable, but the near sterility of F₁s prevented us from obtaining sufficient seed in the BC₁ generation. The BC₂ population was obtained by crossing a single individual of *H. annuus* (ANN1295, 0.4 km from the northern city limits of Hanksville, UT) with a single individual of *H. petiolaris* (PET1277, collected along Highway 89 ≈ 16 km south of Paige, AZ), followed by two generations of backcrossing toward PET1277, as described in more detail by Lexer *et al.* (2003). Because wild sunflowers are self-incompatible, a different individual of *H. petiolaris* was used as the maternal backcross parent in each generation.

Approximately 300 BC₂ seedlings were propagated in Indiana University greenhouses under standard conditions, along with 20 seedlings each of *H. annuus*, *H. petiolaris* and *H. paradoxus*, as described in Lexer *et al.* (2003). After approximately one month, leaf tissue was collected from 172 seedlings (many seedlings were too small for DNA extraction). All seedlings were acclimated with respect to UV, wind, daily temperature changes, low air humidity and elevated NaCl concentrations (repeated treatments with 10 mM NaCl), and then transplanted into *H. paradoxus* salt marsh habitat in May 2001 (Lexer *et al.* 2003). Phenotypic analyses of the 20 replicates per species confirmed that the chosen field site was indeed suitable habitat for the natural hybrid species, *H. paradoxus*, but not for its parents: neither of the two parental species was alive at the end of the experiment, whereas >90% of *H. paradoxus* plants survived (data presented in detail in Lexer *et al.* 2003).

Adaptive trait and fitness measurements in the wild

Candidate adaptive traits and fitness characters were measured in *H. paradoxus* hybrid habitat at Bitterlake National

Wildlife Refuge (US Fish and Wildlife Service) near Roswell, New Mexico (latitude: 33.468369 N; longitude 104.430243 W). The soil at the field site was characterized by high concentrations of Na at the time of transplantation (13 400 ± 2970 ppm) as well as high Ca, Mg and S concentrations (Lexer *et al.* 2003). The seedlings were transplanted within 20 blocks with equal plant numbers per block, thus allowing us to adjust trait measurements for possible environmental variation (below). A small proportion of plants (<2%) died from transplantation or salt shock within the first two days, and these plants were not included in trait or fitness measurements. Adaptive trait and fitness measurements were obtained for 254 BC₂ hybrids transplanted into the field (Lexer *et al.* 2003). However, our study is based on those 172 plants for which sufficient material for DNA extraction was available.

Six elemental uptake traits were measured in the field: Na, S, Mg, Ca, K and B uptake. All traits were measured on a percentage scale in above-ground plant tissue using inductively coupled argon plasma (ICAP) spectrometry (Midwest Laboratories, Inc.), as described previously (Lexer *et al.* 2003). All the traits were significantly associated with fitness (survivorship) in this extremely saline habitat. Briefly, Ca uptake was shown to have a significant *positive* effect on fitness, whereas all other elements including Na and K were *negatively* correlated with fitness. Also, extensive multivariate trait correlations were observed among Na, S, Mg and B (Lexer *et al.* 2003). Two additional traits were measured in the course of the experiment, leaf succulence (% water in leaves) and leaf shape (leaf length/width). However, these were not included in this study, as they were measured in the greenhouse prior to transplantation.

Survivorship in days, relative growth rate in the field and flower number were recorded as potential fitness proxies for each plant. Of these, survivorship was chosen as a fitness character, because: (i) variation in relative growth rates in the salt marsh was low; (ii) data on flower number could be collected for only a small number of plants; and (iii) *H. paradoxus* and its parental species are not expected to differ in survivorship under optimal growing conditions (e.g. when all three species are grown under stress-free conditions in the greenhouse; Welch & Rieseberg 2002).

All traits were tested for deviations from normality using the Kolmogorov–Smirnov test with Lilliefors correction as implemented in SPSS (SPSS Inc.). Where necessary, non-normal traits were transformed using the Box–Cox method (Box & Cox 1964) as implemented by JMP (SAS Institute). Subsequently, all traits were tested for environmental variation by one-way ANOVAs with blocks as the main effect. Two traits, Mg and Na uptake, showed significant variation among blocks. To control for environmental variation in these two traits, further analyses were performed on the

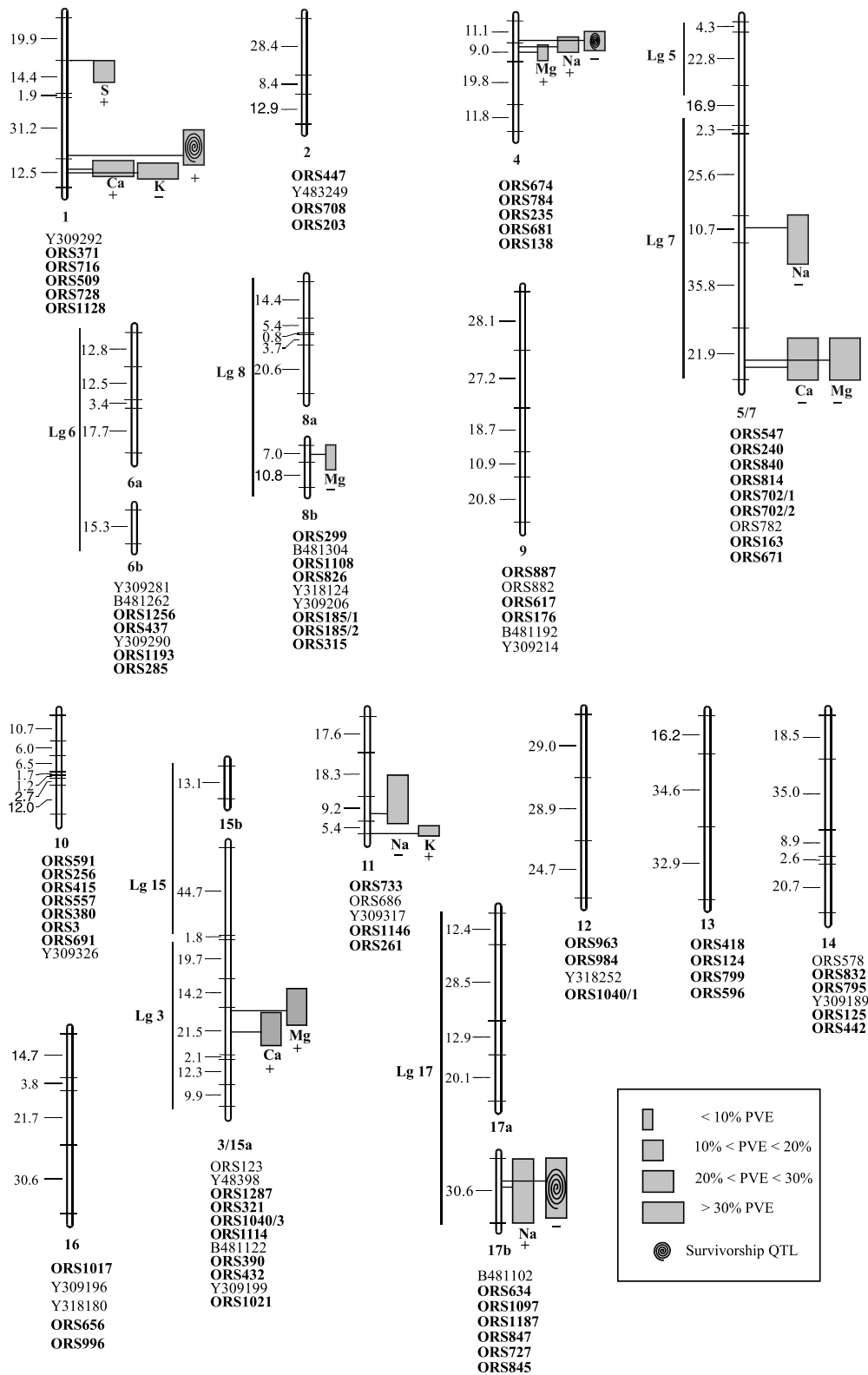


Fig. 1 Linkage map derived from a second generation backcross population (BC_2) of *Helianthus annuus* \times *H. petiolaris*, and QTL positions for survivorship and five elemental uptake traits measured in the wild. Marker positions are shown by horizontal lines, and map distances between markers by 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, and microsatellite markers chosen for this experiment are given in bold. Marker names starting with ORS refer to microsatellites isolated from *H. annuus* (Tang *et al.* 2002), the remaining names refer to AFLPs (Rieseberg *et al.* unpublished).

residuals from the one-way ANOVA, rather than on the original trait values.

Interspecific molecular linkage map

The molecular markers used were chosen from an existing linkage map constructed from 384 individuals of the same interspecific BC₂ cross. These plants formed part of a large QTL mapping experiment conducted in the greenhouse (Rieseberg *et al.* unpublished). The original QTL framework map consisted of 96 markers (Fig. 1) comprising 76 microsatellites and 20 amplified fragment length polymorphisms (AFLP; Vos *et al.* 1995). Linkage group assembly was based on recombination estimates in a BC₂ model using MAP MANAGER QTX Version b16 (Manly *et al.* 2001). The best local order of markers within groups was determined by maximizing the sum of adjacent LOD scores, and map distances in centimorgans (cM) were obtained using the Kosambi mapping function (Kosambi 1944). All 17 linkage groups of sunflower were recovered in the interspecific BC₂, and group names were assigned according to existing microsatellite maps for *H. annuus* (Burke *et al.* 2002; Tang *et al.* 2002). The interspecific BC₂ map showed several cases of pseudolinkage and/or fragmentation (Fig. 1), two phenomena that are often observed in interspecific hybrid crosses (Livingstone *et al.* 2000).

For our study, 71 microsatellite markers were chosen from our interspecific map (Fig. 1, marker names in bold type). This allowed us to examine 52 marker intervals spanning 970 cM (77.5%) of the original map, with an average distance of 13.7 cM between two adjacent markers. Comparisons with the estimated map length of *H. annuus* (1650 cM; Gentzbittel *et al.* 1995) may be inappropriate because of pseudolinkage, fragmentation (Fig. 1), and suppressed recombination in the interspecific BC₂ (not shown).

DNA isolations and genotyping

Total genomic DNA was isolated from \approx 100 mg of dried leaf tissue using the DNeasy plant mini kit (Qiagen) and quantified using a TKO-100 fluorometer (Hoefer Scientific Instruments). DNA was obtained from 172 BC₂ individuals, and the plants were genotyped for 71 microsatellite loci selected from the interspecific map (above), following the protocols of Burke *et al.* (2002). The genotypes were resolved on a ABI 3700 automated sequencer (Applied Biosystems). Up to seven polymerase chain reaction (PCR) products were multiplexed in each lane by running separate PCRs and combining the reaction products. Molecular sizes in base pairs were determined using the GENESIZE R500 ROX size standard (GenPak), and the result files from the ABI 3700 were analysed using GENESCAN 3.5 and GENOTYPER 3.6 (Applied Biosystems). Only microsatellite alleles from

H. annuus were scored because many of the alleles from the *H. petiolaris* did not amplify reliably. Because this is a backcross mapping population, however, no useful genetic information is lost.

The majority of the 71 primer pairs amplified easily interpretable, single-locus banding patterns. A small number of primers produced more complex banding patterns, apparently amplifying multiple, unlinked loci, as indicated in Fig. 1 (e.g. ORS 185/1 and ORS 185/2).

QTL and selection analysis

QTL analyses of six adaptive candidate traits and survivorship were conducted using composite interval mapping (CIM; Zeng 1993, 1994) as implemented in the program MAP MANAGER QTX (Manly *et al.* 2001). This method tests the hypothesis that a QTL is present in an interval between two adjacent markers, while at the same time controlling for the effects of segregating QTLs elsewhere in the genome. The method is essentially identical to that implemented in the program QTL CARTOGRAPHER (Basten *et al.* 1994, 2001), except that markers used as cofactors in CIM are selected manually on the basis of linear marker regressions. MAP MANAGER QTX was chosen because it is able to resolve incomplete genotypes with the help of adjacent markers, and because it combines both linkage mapping and QTL analysis in a BC₂ model within one program. Tests were performed at 1 cM steps, and five background markers were included as cofactors in each CIM model. In general, unlinked markers were chosen on the basis of linear marker regressions, in order to control for background genetic variation elsewhere in the genome and to increase the power of QTL detection (Jiang & Zeng 1995). Whenever the likelihood ratio (LR) profile along a particular group suggested the presence of more than one QTL, cofactors were also selected from the group in question, in order to increase the precision of QTL localization and to detect possible false-positive 'ghost' QTL.

Genome-wide threshold values for declaring the presence of QTLs were determined by 1000 permutations for each trait (Churchill & Doerge 1994), as implemented by MAP MANAGER QTX. One-LOD support limits for the position of each QTL were calculated from the CIM results. For two QTLs the LR profiles were incomplete, because they were located at the end of the chromosomal segment tested (S uptake on Lg1), or at the end of an entire linkage group (K uptake on Lg11). However, because both QTLs were also detected in single marker regressions ($P < 0.005$), we chose to include them in our results (Table 1, Fig. 1). Composite interval mapping in MAP MANAGER QTX was also employed to estimate the additive effect of each QTL, as well as QTL magnitudes expressed as the percent phenotypic variation explained (PVE) in the BC₂. As an

Table 1 Putative QTL positions, likelihood ratios (LR), significance levels, per cent phenotypic variance explained (PVE) and additive effects for five elemental uptake traits as well as survivorship in a second generation backcross population (BC₂) of *Helianthus annuus* × *Helianthus petiolaris*, transplanted into the highly saline habitat of the natural hybrid species *H. paradoxus*

Trait	Linkage group*	Position (cM)	Interval markers	LR	Significance	PVE (%)	Additive effect
Na	4	12†	ORS784 — ORS235	16.0	< 0.01	15	+1.88
	7	76†	ORS702/2§ — ORS163	15.3	< 0.05	17	-2.39
	11	42†	ORS733§ — ORS1146	15.0	< 0.05	15	-2.67
	17b	13†	ORS727§ — ORS845	15.6	< 0.05	18	+1.88
Ca	1	70†	ORS728 — ORS1128	17.0	< 0.01	32	+1.36
	3	90†	ORS1114 — ORS390	20.7	< 0.005	19	+1.91
	7	135‡	ORS163 — ORS671	14.1	< 0.05	27	-1.04
Mg	3	81†	ORS1114 — ORS390	15.3	< 0.05	13	+0.46
	4	15‡	ORS784 — ORS235	23.5	< 0.005	17	+0.31
	7	132‡	ORS163 — ORS671	12.3	< 0.05	23	-0.34
	8b	4‡	ORS185/1 — ORS185/2	14.7	< 0.05	10	-0.18
K	1	73†	ORS728 — ORS1128	19.7	< 0.005	36	-3.29
	11	49†	ORS261	15.8	< 0.05	14	+1.73
S	1	20†	ORS371	14.9	< 0.05	21	+0.36
Survivorship	1	66†	ORS509 — ORS728	15.5	< 0.05	11	+0.63
	4	10†	ORS674 — ORS784	16.1	< 0.005	11	-0.52
	17b	10†	ORS727§ — ORS845	17.9	< 0.005	16	-0.52

*Linkage group designation according to genetic maps of *Helianthus annuus* (Burke *et al.* 2002; Tang *et al.* 2002).

†QTLs exclusively expressed in the field.

‡QTLs also detected in the greenhouse (Rieseberg *et al.* unpublished).

§Markers displaying significant segregation distortion ($P < 0.05$ corrected for multiple tests).

alternative method of estimating QTL magnitudes, the additive effect of each QTL was scaled relative to the species differences between *H. annuus* and *H. petiolaris* transplanted into the salt marsh. Also, segregation distortion was analysed for all markers linked to a QTL. This was necessary because segregation distortion may potentially bias QTL detection and effect estimation. For each marker, observed marker frequencies were compared to Mendelian expectations in the BC₂ by χ^2 goodness-of-fit tests, and P -values were adjusted for multiple tests (Rice 1989).

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. Our selection estimates were for heterozygous BC₂ genotypes carrying a marker allele derived from *H. annuus*. Following Hedrick (2000; p. 97), s was calculated as $(w_{12} - 1)/2$ where w_{12} is the relative fitness of the heterozygous genotype class. Because the degree of dominance cannot be tested in a backcross breeding design, gene action was assumed to be purely additive ($h = 0.5$).

Results

Candidate adaptive trait and survivorship QTLs expressed in the wild

A total of 14 elemental uptake QTLs and three survivorship QTLs were detected in our BC₂ mapping population

transplanted into *Helianthus paradoxus* salt marsh habitat (Table 1; Fig. 1). Between one and four QTLs were detected for each of the elemental uptake characters, except for boron uptake, for which no significant QTLs were found. Taken together, the QTLs identified for elemental uptake were able to explain 65% (Na uptake), 78% (Ca uptake), 63% (Mg uptake), 50% (K uptake) and 21% (S uptake) of the phenotypic variation in these traits in the BC₂ population. Three of these elemental uptake QTLs were shown to have major phenotypic effects (PVE > 25%). In addition, three QTLs were identified that cumulatively explain 38% of the variation in survivorship in this extreme habitat. It is likely that we overlooked some smaller QTLs in this experiment, however, as our sample size of 172 BC₂ plants provides only limited power to detect QTLs with small effects (Lynch & Walsh 1998). The small sample size also likely created an upward bias in the estimation of QTL magnitudes (Beavis 1994).

Note that there is considerable debate regarding the most appropriate method for estimating QTL magnitudes. QTL mapping may upwardly bias estimates of QTL magnitudes, because genome-wide scans are a joint estimation of QTL position and size (Goering *et al.* 2001). Also, our method of expressing QTL magnitudes relative to the phenotypic variation in the BC₂ (PVEs; Table 1) is not the only one. Alternative methods include scaling of additive effects relative to the proportion of population differences explained or to the proportion of 'standing variation' in the

background population (e.g. Fishman *et al.* 2002). The 17 QTLs detected here explain on average 88% of the species differences between our control groups of *H. annuus* and *H. petiolaris* transplanted into the salt marsh (effect sizes ranging from 20 to 160%). These surprisingly high estimates likely stem from cryptic genetic variation (QTLs of opposing effects) in the two parental species.

Ten of the 14 elemental uptake QTLs expressed in the salt marsh habitat were not detected in a recent greenhouse QTL experiment involving the same BC₂ cross (Rieseberg *et al.* unpublished). These 10 QTLs include the two largest QTLs detected in the wild, Ca uptake and K uptake on linkage group 1, explaining 32 and 36% of the phenotypic variation in the interspecific BC₂ (Table 1, Fig. 1). The finding that the majority of the QTLs detected in *H. paradoxus* hybrid habitat are expressed exclusively in the wild is congruent with earlier results, in which pronounced differences in phenotypic trait expression were observed for elemental uptake between the salt marsh and the greenhouse (Welch & Rieseberg 2002; Lexer *et al.* 2003).

Visual examination of the distribution of additive effects for each trait reveals that QTLs with both positive and negative effects were present for five of the traits studied (Table 1). This pattern could not be tested for S uptake and B uptake, as only one QTL was identified for S, and none for B uptake. The presence of QTLs with positive and negative effects in interspecific BC₂ hybrids between *H. annuus* and *H. petiolaris* may have important implications for the origin of ecological divergence in the natural hybrid species, *H. paradoxus* (see Discussion).

Co-localization of adaptive trait and survivorship QTLs

QTL analysis allowed us to examine whether QTLs for elemental uptake and QTLs controlling survivorship in *H. paradoxus* hybrid habitat might be the same. All three survivorship QTLs mapped to the same genomic regions as QTLs for Ca and K uptake (Fig. 1, linkage group 1), Na and Mg uptake (Fig. 1, linkage group 4) or Na uptake (Fig. 1, linkage group 17b). In all three cases, the one-LOD support intervals of elemental uptake and survivorship QTLs overlapped, and the microsatellite markers closest to the LR peaks were the same for both classes of QTLs (Fig. 1). Thus, we appear to have identified three genomic regions associated with ecological selection in the wild.

The directions of additive QTL effects on linkage groups 1, 4 and 17b provide information about the possible functional effects of elemental uptake traits on fitness in the wild. On linkage group 1, a major QTL that increased Ca content, and a tightly linked or pleiotropic QTL that decreased K content, were associated with prolonged survivorship in the salt marsh (Fig. 1). The same region also had a characteristic, although nonsignificant, negative

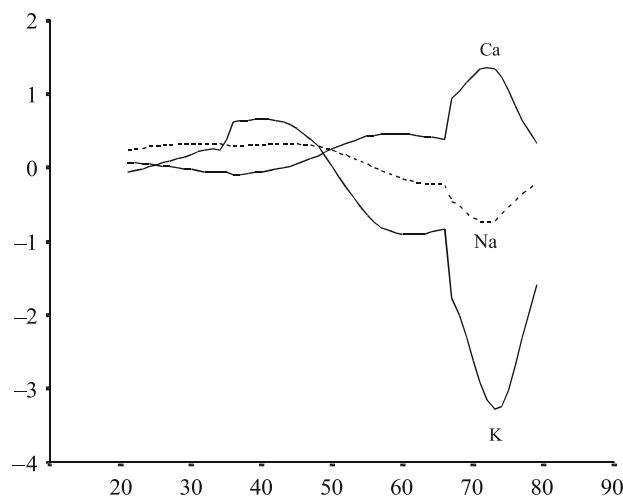


Fig. 2 Additive effects for Ca, K and Na uptake along a 60-cM segment of linkage group 1. Map distances are given on the x-axis, and the additive effects for Ca and K uptake (solid lines) and Na uptake (dashed line) are given on the y-axis. For details see text.

effect on Na uptake (Fig. 2). On linkage group 4, two tightly linked or pleiotropic QTLs that increased Na and Mg content were negatively associated with survivorship in the salt marsh. Likewise, a QTL that increased Na uptake on linkage 17b was negatively associated with survivorship.

Strength of selection for adaptive QTLs

The strength of natural selection for elemental uptake QTLs on linkage groups 1, 4 and 17b was examined by calculating selection coefficients (*s*) for heterozygous BC₂ 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, -0.084 for the correlated QTLs controlling Mg and Na uptake on linkage group 4 and -0.094 for the Na uptake QTL on linkage group 17b (Fig. 3).

These selection estimates are not biased upward by viability selection unrelated to salt tolerance: of a total of 17 QTLs detected in this study, only 4 were associated with markers showing segregation distortion (Table 1), among them only one survivorship QTL (Table 1; selection coefficient in Fig. 3C). The *H. annuus*-derived marker allele in this case (marker ORS 727) was *over-represented* in the BC₂ ($P < 0.001$), whereas selection in the wild was in a *negative* direction (Fig. 3C). Hence, segregation distortion may have led us to *underestimate* the strength of ecological selection in Fig. 3(C). For the remaining two genomic regions under selection in the wild (Fig. 3A, B), no segregation distortion was detected.

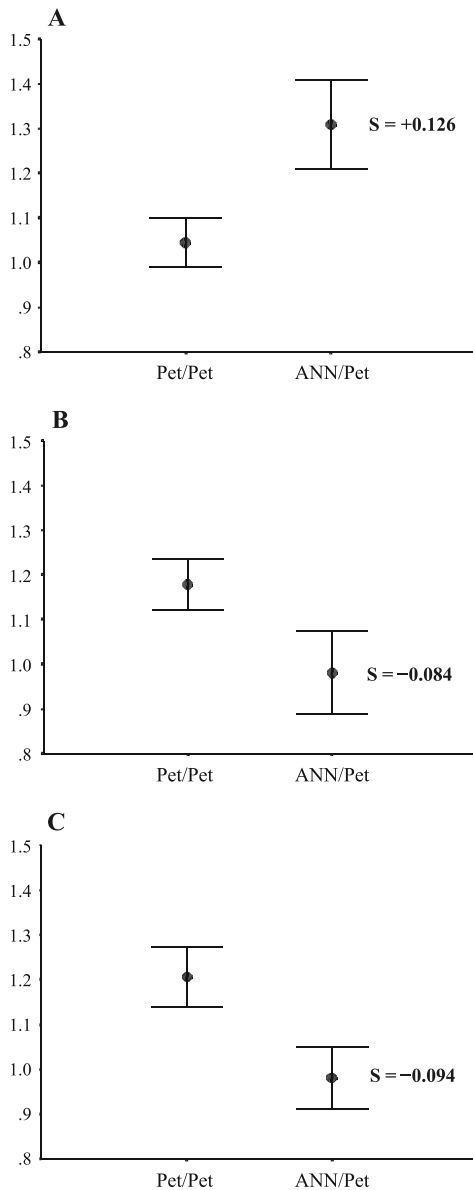


Fig. 3 Fitness differences among genotypic classes and selection coefficients for three microsatellite markers that showed significant marker–fitness associations. Fitness means \pm SE (fitness = relative survivorship) are given for *H. petiolaris* homozygotes (Pet/Pet) and heterozygous individuals displaying a *H. annuus* marker band (ANN/Pet). Selection coefficients for the heterozygous class are indicated in bold. (A) Linkage group 1, ORS728; (B) linkage group 4, ORS784; (C) linkage group 17b, ORS727.

Discussion

Origin of ecological divergence in homoploid hybrid taxa

Theoretical studies of homoploid hybrid speciation identify niche separation between the hybrid neospecies

and its parents as the most important factor favouring hybrid establishment (McCarthy *et al.* 1995; Buerkle *et al.* 2000). Without niche differentiation, new hybrid genotypes are likely to be overcome by competition and/or gene flow from parental populations. In accordance with these predictions, most (perhaps all) stabilized introgressants or hybrid species are ecologically divergent with respect to their parental species (Abbot 1992; Arnold 1997; Rieseberg 1997; Johnston *et al.* 2001; Schwarzbach *et al.* 2001; Rosenthal *et al.* 2002). A major question concerns how this divergence arises.

The possibility that has received the most attention is transgressive segregation, which refers to the generation of extreme phenotypes in segregating hybrid populations (deVicente & Tanksley 1993; Rieseberg *et al.* 1999). Transgressive segregation is a frequent characteristic of hybrids (Rieseberg *et al.* 1999) and has a simple genetic basis (deVicente & Tanksley 1993). If parental taxa are fixed for alleles with opposing effects within species, then extreme phenotypes are the predicted result of recombination in segregating hybrid populations (i.e. complementary gene action). It has been hypothesized that the niche separation that is so crucial for homoploid hybrid speciation arises through selection for extreme or transgressive phenotypes in the ancestral hybrid population, but this has not yet been shown.

In the present study we provide important new evidence in support of this hypothesis. In particular, all mineral ion uptake traits with more than one detected QTL had QTL effects in opposing directions. Thus, as in domesticated plants, complementary gene action appears to be responsible for the generation of extreme phenotypes in hybrid sunflower populations. This genetic architecture provides an ideal substrate for rapid ecological divergence in hybrid neospecies and offers a simple explanation for the colonization of salt marsh habitats by *Helianthus paradoxus*. The transgressive segregation hypothesis is further strengthened by the detection of QTLs that have opposing effects on fitness (i.e. survivorship). This means that in some instances, QTL alleles from *H. annuus* increase survivorship (linkage group 1), whereas in other instances, *H. annuus*-derived alleles decrease survivorship, i.e. it is the *H. petiolaris* QTL allele that is adaptive (linkage groups 4 and 17b). This result confirms the long-held view that hybridization may serve as a source of genetic variation upon which selection can act (Anderson 1949; Stebbins 1959; Lewontin & Birch 1966; Arnold 1997; Barton 2001).

Genetic dissection of a correlational selection response

Uptake of Ca and other mineral ions is positively correlated in hybrids of *H. annuus* \times *H. petiolaris*, yet colonization of *H. paradoxus* hybrid habitat appears to require a negative correlation between uptake of Ca and other mineral ion

traits. Our previous analysis of selection on mineral ion uptake traits (Lexer *et al.* 2003) revealed that the positive correlation between Ca and other mineral ions decreased over time, suggesting that it may be feasible to reduce or eliminate this correlation by selection. The QTL analyses described here establish the genetic basis for this correlation and offer an explanation for its reduction over time.

There are three QTLs for Ca uptake and all three map to positions that overlap with those of other mineral ion uptake QTLs. In two instances (linkage groups 5/7 and 3/15), Ca uptake is positively correlated with the uptake of other mineral ions, indicating that tight linkage and/or pleiotropy accounts, at least in part, for the positive trait correlations observed. For the remaining Ca uptake QTL (linkage group 1), Ca is negatively correlated with other mineral ion uptake traits (Figs 1 and 2). This QTL also happens to have a significant effect on survivorship. It is easy to see how an increase in the frequency of this QTL would lead to a reduction in the correlation between the uptake of Ca and other mineral ion traits and this is exactly what happened during the selection experiment. Note that several other chromosomal segments exhibit a similar negative correlation between Ca uptake and that of other mineral ions, but their effects were too small to achieve significance at genome-wide threshold levels.

Selection coefficients and the likelihood of homoploid hybrid speciation

For speciation to occur in the presence of gene flow, as is likely to be the case for homoploid hybrid speciation, the strength of divergent selection on individual loci (s) must exceed the migration rate (m). Although m is not precisely known for annual sunflowers, $N_e m$ values are typically < 1 (Schwarzbach & Rieseberg 2002). Thus, the selection coefficients reported here (0.084–0.126) are sufficiently large to enable divergence in sympatry as long as the size of the ancestral population for *H. paradoxus* exceeds 12 individuals, which seems likely. For larger populations, such as those typical of wild sunflowers, the selection coefficients observed here are far larger than required for divergence in the presence of gene flow (for $N_e = 100$, s must exceed 0.01; for $N_e = 1000$, s must exceed 0.001, and so forth). Thus, the magnitude of selection coefficients required for the hybrid origin of *H. paradoxus* is probably considerably lower than those reported here.

Linkage vs. pleiotropy — functional considerations

The colocalization of QTLs for elemental uptake and survivorship, and the directions of their additive effects, allow us to speculate about the functional effects of elemental uptake QTLs on fitness in the wild. On linkage group 1, for example, major QTLs for increased Ca and

decreased K uptake, respectively, were highly correlated with a QTL that prolonged survivorship (Fig. 1). We do not know at present whether this genetic correlation is due to tight linkage or pleiotropy, as the limited number of recombination events accumulated in our BC₂ line prevents fine-mapping of the QTLs (Lynch & Walsh 1998). However, existing knowledge about the nature of elemental uptake from the soil, and about the functional role of these elements in salt adaptation in plants, suggests that the observed correlations arose because of pleiotropy, i.e. uptake of multiple elements and fitness in the salt marsh are controlled by overlapping sets of genes.

Accumulating evidence for pleiotropy stems from the molecular salt tolerance literature. It is now widely accepted that Ca plays an important role in salt stress response in plants, because it can ameliorate the adverse effects of Na in many ways (Volkmar *et al.* 1997). In particular, high Ca levels have been shown to result in greater salt tolerance by Na exclusion, because Ca ions are capable of mediating changes in the activity, and selectivity, of transport systems that are shared by Na and K ions (Hasegawa *et al.* 2000b). Also, much attention has been paid to the more general role of Ca in signalling during abiotic stress response (Sanders *et al.* 1999; Knight 2000). Alterations in cytosolic Ca levels are known to constitute a signal that is transduced via Ca-dependent proteins to affect a wide array of downstream responses involved in the protection of the plant, ultimately resulting in adjustment to abiotic stresses (Knight 2000). Pleiotropic effects of QTLs that increase Ca levels and at the same time decrease the content of other elements are consistent with both of these molecular findings, and it is also likely that pleiotropic effects of these QTLs are responsible for the observed differences in survivorship.

Interestingly, the same genomic region on linkage group 1 that harbours QTLs for Ca and K uptake also had a negative (although nonsignificant) effect on Na uptake (Fig. 2). This is consistent with our hypothesis that the survivorship QTL located in this region may stem, at least in part, from pleiotropic effects of Ca uptake on Na exclusion. Also, pleiotropic effects of decreased K uptake on Na exclusion are plausible, as both elements are present as monovalent ions in the soil, and both share overlapping sets of transport molecules (Volkmar *et al.* 1997; Hasegawa *et al.* 2000b). Indeed, many studies on salt tolerance in plants evaluate K/Na ratios rather than each element individually, as the capacity to maintain a high cytosolic ratio of these two ions may be a key element of salt tolerance (Yeo 1998). However, we chose to analyse each ion separately, because their ratios deviated strongly from normality. A more accurate dissection of K and Na uptake may be possible once candidate genes for high-affinity K uptake vs. low-affinity K/Na uptake are genetically mapped relative to elemental uptake QTLs (see below).

In a similar way, it is possible to interpret the genetic correlations observed among Na uptake, Mg uptake and survivorship on linkage group 4 (Fig. 1). On this group, major QTLs that increased Na and Mg content were associated with a QTL that decreased survivorship in the salt marsh. This finding is consistent with the fact that both Na and Mg uptake were strongly negatively correlated with fitness in the same transplantation experiment (Lexer *et al.* 2003). Obviously, increased salinity tolerance in the more fit BC₂ plants was achieved by QTLs conferring Na exclusion, and the same appears to be true for Mg, which was present in very high, and presumably toxic, concentrations in the field soil (Lexer *et al.* 2003).

Note that changes in elemental concentrations, such as those observed here, have sometimes been attributed to QTLs involved in vegetative growth (e.g. Koyama *et al.* 2001). However, this is unlikely to be the case here, because variation in growth rates in the BC₂ was low, and elemental concentrations were *not* correlated with growth in the field. Hence, elemental concentrations in BC₂ hybrids appear to be independent of growth and likely reflect true elemental uptake from the soil.

Our results have important implications for the identification of candidate genes involved in the evolution of salt adaptation in early generation hybrids between *H. annuus* and *H. petiolaris*. Expressed sequence tag (EST) libraries have been constructed and sequenced for annual sunflowers (<http://cgpdb.ucdavis.edu/>), thereby allowing the actual genes underlying adaptive QTL variation in wild sunflower hybrids to be isolated and characterized. Results from our transplantation experiment suggest that Ca-dependent salt tolerance genes are of particular interest. These may include genes involved in Ca fluxes across membranes, such as Ca pumps or carriers, Ca sensors, such as Ca-dependent protein kinases, or potential targets of Ca signals, such as K/Na ion channels with varying specificity (Sanders *et al.* 1999; Hasegawa *et al.* 2000a,b; Knight 2000). Mapping of candidate Ca-dependent salt tolerance ESTs in our BC₂ population is underway.

Acknowledgements

We thank J. Malcom and G. Warrick (US Fish and Wildlife Service) for their assistance during field work, as well as K. Livingstone and O. Raymond of the Rieseberg laboratory for their help during the initial stages of genetic map construction. This study was supported by Erwin-Schrodinger grant J-2148 of the Austrian Science Foundation to CL, and by NIH grants R01 G59065 and T32 G07705-21.

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The laboratory of Loren Rieseberg studies the genetics of speciation in plants. This study, which was part of Christian Lexer's postdoctoral research, contributes to an ongoing research programme on the origin of novel adaptation in three wild, annual sunflower hybrid species. Mark Welch, who has recently finished his PhD thesis in the Rieseberg laboratory, is currently a post-doctoral researcher at Vanderbilt University, Nashville, TN. Jennifer Durphy works as a technician in the Rieseberg laboratory, in addition to pursuing a career as an oboist.
