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Extensive Chromosomal Repatterning and the Evolution of Sterility Barriers in Hybrid Sunflower Species

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ABSTRACT

New species may arise via hybridization and without a change in ploidy. This process, termed homoploid hybrid speciation, is theoretically difficult because it requires the development of reproductive barriers in sympatry or parapatry. Theory suggests that isolation may arise through rapid karyotypic evolution and/or ecological divergence of hybrid neospecies. Here, we investigate the role of karyotypic change in homoploid hybrid speciation by generating detailed genetic linkage maps for three hybrid sunflower species, *Helianthus anomalus*, *H. deserticola*, and *H. paradoxus*, and comparing these maps to those previously generated for the parental species, *H. annuus* and *H. petiolaris*. We also conduct a quantitative trait locus (QTL) analysis of pollen fertility in a BC_2 population between the parental species and assess levels of pollen and seed fertility in all cross-combinations of the hybrid and parental species. The three hybrid species are massively divergent from their parental species in karyotype; gene order differences were observed for between 9 and 11 linkage groups (of 17 total), depending on the comparison. About one-third of the karyoypic differences arose through the sorting of chromosomal rearrangements that differentiate the parental species, but the remainder appear to have arisen *de novo* (six breakages/six fusions in *H. anomalus*, four breakages/three fusions in *H. deserticola*, and five breakages/five fusions in *H. paradoxus*). QTL analyses indicate that the karyotypic differences contribute to reproductive isolation. Nine of 11 pollen viability QTL occur on rearranged chromosomes and all but one map close to a rearrangement breakpoint. Finally, pollen and seed fertility estimates for F_1's between the hybrid and parental species fall below 11%, which is sufficient for evolutionary independence of the hybrid neospecies.

OVER 90% of plant and animal species differ in their karyotypes (White 1978; King 1993). Many karyotypic differences are likely to be incidental to speciation, either because they arose after reproductive isolation was complete or because they have little impact on hybrid fertility (*e.g.*, John 1981; Coyne *et al.* 1991,1993) or recombination rates (Rieseberg 2001). However, some chromosomal rearrangements may trigger speciation (Levin 2002), particularly when geographical barriers to gene flow are absent. In these situations, the rearrangements may decrease gene flow sufficiently among diverging populations to allow selected differences or hybrid incompatibilities to accumulate (Rieseberg *et al.* 1999; Noor *et al.* 2001; Navarro and Barton 2003; Brown *et al.* 2004). Chromosomal rearrangements may impede gene flow by causing a reduction in hybrid viability or fertility (Barton and Bengtsson 1986; King 1993) and/or a reduction in recombination among the rearranged chromosomes (Noor *et al.* 2001; Machado *et al.* 2002; Navarro and Barton 2003).

It is not yet clear which of these mechanisms (reduced hybrid fertility *vs.* reduced recombination) most commonly contributes to chromosomal speciation, but models that treat chromosomal rearrangements as recombination modifiers (Noor *et al.* 2001; Rieseberg 2001) have stronger theoretical support. Mathematical analyses indicate that hybrid incompatibilities arise several times faster in regions of low recombination than in other genomic regions (Navarro and Barton 2003). However, these models are mostly applicable to inversions, which are known to greatly reduce effective recombination rates (Greenbaum and Reed 1984; Hale 1986; Navarro and Ruiz 1997; Coyne *et al.* 1993). In contrast, translocations and other kinds of rearrangements have smaller effects on recombination rates (although, see Davisson and Akeson 1993).

Chromosomal speciation models that rely on a reduction in hybrid fertility generally have less theoretical support than recombination modifier models because rearrangements that strongly reduce heterozygote fitness (*i.e.*, underdominance) are unlikely to be fixed

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through drift, except in small, inbred populations (Hedrick 1981; Walsh 1982; Lande 1985). Weakly underdominant mutations are more easily established, but will not be very effective as reproductive barriers.

Several solutions to this paradox have been suggested. In chain or cascade models, strong isolation arises following the accumulation of weakly underdominant rearrangements (White 1978). Other models posit a scenario in which geographically isolated populations become fixed for different rearrangements (*e.g.*, centric fissions) that have no effect on fertility on their own, but are strongly underdominant in combination (Baker and Bickham 1986). A third solution—recombinational speciation—invokes interspecific hybridization as a mechanism for karyotypic evolution; reproductive isolation is achieved via the sorting of chromosomal rearrangements that differentiate the parental species (Grant 1981) or by rearrangements induced by recombination (Templeton 1981; Rieseberg *et al.* 1995). Because rearrangement polymorphisms often are initiated at high frequencies in hybrid populations, establishment of a new homokaryotype through drift poses fewer theoretical hurdles than in nonhybrid populations (McCarthy *et al.* 1995; Pialek *et al.* 2001), particularly when there is ecological and spatial isolation between the hybrid derivative and its parental species (Buerkle *et al.* 2000).

This article focuses on the third mode of chromosomal speciation, recombinational speciation, using the annual sunflowers of the genus Helianthus as a study system. This group is well suited for the study of recombinational speciation because three of 11 species (*Helianthus anomalus*, *Helianthus deserticola*, and *Helianthus paradoxus*) are stabilized diploid hybrid derivatives of the same two parental species (*Helianthus annuus* and *Helainthus petiolaris*; Rieseberg 1991). Furthermore, meiotic analyses suggest that the parental and hybrid species differ significantly in chromosome structure (Chandler *et al.* 1986), an observation consistent with the recombinational model. Genetic map-based comparison of one of the hybrid species (*H. anomalus*) with that of its parents indicates that hybrid speciation was accompanied by massive karyotypic change (Rieseberg *et al.* 1995), as predicted by the earlier meiotic studies.

Here we extend these earlier comparative mapping studies to include the two other hybrid species, *H. deserticola* and *H. paradoxus*. We also provide a microsatellite map for *H. anomalus*, which provides significant additional resolution relative to the previously published RAPD map for this species (Rieseberg *et al.* 1995) and enables direct comparisons with the newly developed maps for *H. deserticola* and *H. paradoxus*. Microsatellite maps of the three hybrid species are compared to those recently published for the parental species (Burke *et al.* 2004).

In addition to the comparative mapping work, we analyze the fertility effects of the mapped chromosomal rearrangements by conducting a quantitative trait locus (QTL) analysis of pollen viability in a BC_2 population between the parental species, *H. annuus* and *H. petiolaris*. Finally, we report on the pollen and seed fertility of first-generation hybrids from all combinations of crosses between the two parental species and their three hybrid-derivative species.

Results from these three data sets are used to address the following questions/assumptions that underlie the recombinational speciation model:

1. Has karyotypic change accompanied the formation of the three hybrid species as required by the recombinational model and, if so, what is the nature of the karyotypic changes? That is, do the hybrid species simply possess a combination of parental rearrangements, or are there unique rearrangements in the hybrids? Also, do novel rearrangements most commonly involve chromosomes that are already rearranged between the parental species as predicted by theory (Templeton 1981)?
2. Are map lengths greater in the hybrid than in parental species? Grant (1958) argues that high recombination rates will be favored during recombinational speciation.
3. Are chromosomal rearrangements in Helianthus strongly underdominant as assumed by the recombinational model? Specifically, what fraction of the variance in pollen sterility maps to chromosomal rearrangements and how large are the effects of individual translocations and inversions?
4. Are chromosomal sterility barriers between the hybrid and parental species strong enough to allow the hybrid species to evolve independently, even when parapatric with parental species populations (Buerkle *et al.* 2000)?

MATERIALS AND METHODS

Study system: The five Helianthus species analyzed are diploid ($n = 17$), self-incompatible annuals, all native to North America (Figure 1). *H. annuus* and *H. petiolaris*, the parental species, are abundant in the central and western United States, but differ in habitat preference. *H. annuus* occurs in mesic, clay-based soils, whereas *H. petiolaris* is found in drier, sandier soils. The three hybrid species, in contrast, are restricted to extreme habitats in the desert Southwest. *H. anomalus* is found on desert sand dunes in Utah and northern Arizona, *H. deserticola* in more stabilized deposits in the Great Basin Desert (Nevada, Utah, Arizona), and *H. paradoxus* in saline desert wetlands in west Texas and New Mexico (Heiser *et al.* 1969; Rogers *et al.* 1982). Divergence times estimated from microsatellite and chloroplast DNA variation place the divergence of the parental species between 75,000 and 1 million years before present (BP) and the origin of their three hybrid derivatives between 60,000 and 200,000 years BP (Rieseberg *et al.* 1991; Schwarzbach and Rieseberg 2002; Welch and Rieseberg 2002; Gross *et al.* 2003).

The five species appear to be strongly isolated reproductively. All five taxa occur in very different habitats and reciprocal transplant experiments demonstrate adaptive ecological divergence (Lexer *et al.* 2003b; Gross *et al.* 2004; Ludwig *et al.* 2004). Previous mapping studies (Rieseberg

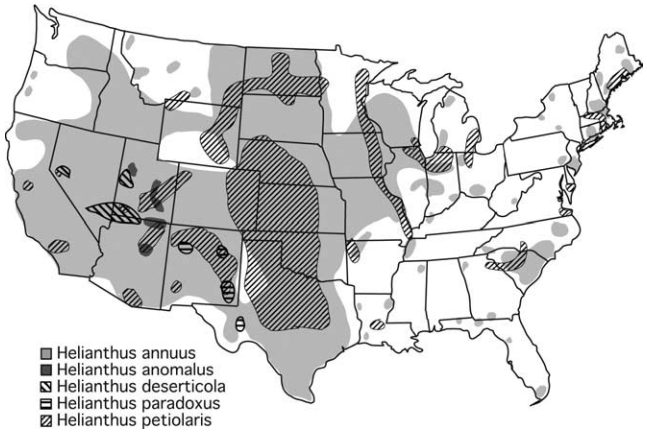


Figure 1.—Present-day distributions of the two parental species, *H. annuus* and *H. petiolaris*, and their three hybrid-derivative species, *H. anomalus, H. deserticola*, and *H. paradoxus* (based on Rogers *et al.* 1982).

et al. 1995; Burke *et al.* 2004) indicate that the genomes of *H. annuus*, *H. anomalus*, and *H. petiolaris* are extensively rearranged and pollen viability of F_1 hybrids from all three cross-combinations is <5% (Rieseberg 2000). Despite these reproductive barriers, contemporary hybrid zones are common between *H. annuus* and *H. petiolaris* (Heiser 1947; Rieseberg *et al.* 1998). In contrast, no natural hybrids have been reported between the three hybrid species and their parents, although hybridization has been detected between *H. anomalus* and *H. deserticola* in the Little Sahara Sand Dunes in Utah, the one location they are known to occur in parapatry.

Genetic mapping: High-resolution genetic linkage maps were generated for each of the three hybrid species (supplementary Figures S1–S3, available at http://www.genetics.org/supplemental/). For *H. anomalus*, crosses were made between ANO 1497 (Mexican Water, AZ) and ANO 1506 (Hanksville, UT) (Rieseberg *et al.* 1995). Likewise, crosses were made between DES 1471 (Beaver Dam, AZ) and DES 1476 (Virgin, UT) for *H. deserticola*, and between PAR 1084 (Fort Stockton, TX) and PAR 1671 (Dexter, NM) for *H. paradoxus*. For each taxon, an intraspecific hybrid from the populations listed above was crossed to an inbred sunflower line (CMSHA89). This crossing design allowed us to monitor the segregation of alleles from each of the ancient hybrid species against a homogenous genetic background. A total of 54, 58, and 57 individuals were used for genetic map construction in *H. anomalus*, *H. deserticola*, and *H. paradoxus*, respectively.

A variety of markers were employed for mapping. For *H. anomalus*, this study added 241 microsatellite and 78 AFLP markers to the previously published 701 AFLP/RAPD marker map of *H. anomalus* (Ungerer *et al.* 1998), bringing the total number of markers mapped to 1019. The *H. deserticola* and *H. paradoxus* maps were generated from 120 microsatellite and 552 AFLP markers (672 markers total) and 174 microsatellite and 597 AFLP markers (771 markers total), respectively. While map positions of parental species-specific AFLP and RAPD markers were previously reported (427, 290, and 325 markers in *H. anomalus*, *H. deserticola*, and *H. paradoxus*, respectively; Rieseberg *et al.* 2003), markers informative for comparative mapping are described here for the first time.

Maps were developed with the computer program Mapmaker Macintosh V2.0 (Du Pont, Wilmington, DE). Markers were divided into groups using LOD scores of >6 and recombination limits of <0.15. Mapmaker's three-point analysis was used to determine likely orders among markers, followed by multipoint analysis to resolve any discrepancies. Marker orders were confirmed using Mapmaker's "ripple" command, and recombination values were converted to map distances using Kosambi's (1944) mapping function.

For comparisons with the parental species, we employed genetic maps previously described for *H. annuus* and *H. petiolaris* (Burke *et al.* 2004). The *H. annuus* map was constructed from four different maps for this species (Gedil *et al.* 2001; Burke *et al.* 2002; Tang *et al.* 2002; Yu *et al.* 2003) using JoinMap version 3.0 (Van Ooijen and Voorrips 2001). The integrated map includes 288 microsatellite markers that were shared across multiple *H. annuus* maps or that were polymorphic in one of the wild species-mapping populations reported here (Figure S4 at http://www.genetics.org/supplemental/). The map presented here is essentially identical to that provided by Burke *et al.* (2004), except several additional markers that are informative in interspecific comparisons have been added. Also, information on marker homology relative to the other four species is provided for all informative markers.

For *H. petiolaris*, an intraspecific hybrid was crossed with an inbred sunflower line (CMSHA89) and 80 progeny segregating for *H. petiolaris* linkage groups were employed for map construction (Rieseberg *et al.* 1995). The map includes 400 RAPD markers and 295 microsatellites and spans 17 linkage groups and 1592 cM. A map based on a subset of the most informative markers and information on marker homology is provided in Figure S5 (available at http://www.genetics.org/supplemental/).

Comparisons of marker orders and genetic map lengths: Homologous genomic regions across maps were identified from the locations of presumably orthologous microsatellite, AFLP, and RAPD markers. Note that homology of the latter have previously been demonstrated by Southern hybridization and/or restriction fragment digestions (Rieseberg 1996). Rearrangements were inferred from differences among maps in the location and linear order of markers. Map lengths were compared across species by analyzing the distance separating the outermost-shared markers for each collinear segment (Burke *et al.* 2004), although several clearly misplaced markers were excluded from the analysis. A pairwise *t*-test was used to identify differences in mean map lengths among species (Table 1).

QTL analyses of pollen viability: The analysis of pollen viability employed a BC_2 population of *H. annuus* × *H. petiolaris* previously described by Rieseberg *et al.* (2003). The population consists of 384 progeny that have been genotyped for 96 molecular markers that provide coverage of all 17 linkage groups. For each BC_2 plant, fresh pollen was harvested from the first flowering head. The pollen was treated with a solution of 30% sucrose and 0.1% 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium (MTT) (Chandler *et al.* 1986) and ~300 grains were scored for staining indicative of pollen viability. Fully darkened grains were considered viable.

Pollen viability QTL were detected by composite interval mapping (CIM; Zeng 1994) as implemented in the program Mapmanager 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 QTL elsewhere in the genome. Tests were performed at 1 cM steps, and five background markers were included as cofactors in each CIM model. Genome-wide threshold values for declaring the presence of QTL were determined by 1000 permutations (Churchill and Doerge 1994). One-LOD support limits for the position of each QTL were calculated from the CIM results, as were the additive effects and percentage of phenotypic variance explained (PVE) by each QTL (Table 3).

Mapmanager QTX also was employed to search for interaction effects or epistasis by testing all pairs of marker loci

TABLE 1

Comparison of map lengths between two parental and three hybrid Helianthus species

Taxa	No. segments compared	Mean dif. (cm)	Std. error (cm)	*t*-ratio	$P > \|t\|$
H. annuus-anomalus	23	−20.59	4.81	−4.28	0.0003*
H. annuus-deserticola	11	−0.09	5.51	−0.02	0.9872
H. annuus-petiolaris	21	−9.27	4.56	−2.03	0.0557
H. annuus-paradoxus	16	−17.9	7.61	−2.36	0.0321
H. anomalus-deserticola	21	18.45	6.59	2.80	0.0110
H. anomalus-paradoxus	20	2.52	4.77	−0.53	0.6041
H. anomalus-petiolaris	20	6.29	5.53	1.14	0.2686
H. deserticola-paradoxus	13	−5.43	3.26	1.67	0.1211
H. deserticola-petiolaris	4	−2.53	15.29	−0.17	0.8793
H. paradoxus-petiolaris	9	17.84	5.93	3.01	0.0168

*Significant after Bonferroni correction for multiple comparisons.

for both main effects and interaction effects for each trait (Table 4). Unlike CIM, tests were performed only at marker loci. A two-stage test for significance was employed because of the large number of comparisons that must be made for each trait and because the significance of an interaction cannot be reliably tested if there is a strong main effect. First, the total effect of the two loci had to have $P \leq 10^{-5}$. This very stringent threshold was recommended by the user manual and is almost identical to thresholds calculated from the "effective number of comparisons" (Cheverud 2000), which takes linkage among markers into account in establishing significance thresholds. Second, the interaction effect itself must have $P \leq 0.01$.

Crossing relationships: Pollen and seed fertility of interspecific crosses involving *H. annuus*, *H. anomalus*, and *H. petiolaris* have previously been reported (Rieseberg 2000). Thus, for the present study, crosses were made between these species and the two remaining hybrid taxa, *H. deserticola* and *H. paradoxus*, as well as between populations of *H. deserticola* and *H. paradoxus*. Interspecific crosses were made in both directions and replicated at least four times (56 total). Crosses employed a minimum of four individuals from one population of each species: *H. annuus* (Hanksville, UT, Rieseberg 1295); *H. anomalus* (Mexican Water, AZ, Rieseberg 1282); *H. deserticola* (Toquerville, UT, Rieseberg 1270), *H. paradoxus* (Grants, NM, Rieseberg 1370), and *H. petiolaris* (Glenn Canyon Recreation Area, UT, Rieseberg 1277).

Although attempts were made to propagate 3 F_1's from each cross (24 per cross combination), there was significant mortality, and 4–10 F_1 plants were analyzed from each cross-combination. For each F_1 plant that survived to reproductive maturity, pollen was harvested from the first flowering head and tested for viability as described above. Seed set was estimated as the percentage of cross-pollinated flowers (*i.e.*, $F_1 \times F_1$) that produced achenes (one-seeded fruits). Means and standard errors were calculated for pollen viabilities and seed set for each cross combination. To explore the relationship between the number of chromosomal differences and hybrid and seed fertility, correlations were calculated for each case and the data were permuted ×1000 to obtain a null distribution for statistical testing.

RESULTS

Map lengths: Seventeen linkage groups were recovered for each of the three hybrid species, which corresponds to their haploid chromosome number (supplementary Figures S1–S3 at http://www.genetics.org/supplemental/). Linkages were labeled according to the standard nomenclature for *H. annuus* (Berry *et al.* 1995; Gedil *et al.* 2001). The relationship between this nomenclature and that of Rieseberg *et al.* (1995, 1999) is shown in Burke *et al.* (2004) and Rieseberg *et al.* (2003). For *H. anomalus*, the 1019 markers spanned 1908.3 cM, with an average spacing between markers of 1.90 cM. Map lengths and average marker spacing are 1229/1.88 cM and 1420.5/1.88 cM for *H. deserticola* and *H. paradoxus*, respectively. In comparison, the lengths of the *H. annuus* and *H. petiolaris* maps are 828 and 1592 cM, respectively (Burke *et al.* 2004).

Despite the seemingly large differences in map lengths, direct comparisons based on distances between orthologous markers indicate that only the longest (*H. anomalus*) and shortest (*H. annuus*) maps differ significantly. Even this result is suspect because the integrated *H. annuus* map employed in these comparisons is substantially shorter than most individual maps for *H. annuus* (Burke *et al.* 2004). Thus, recombinational speciation in Helianthus does not appear to have been accompanied by significant changes in recombination rates, although note that the small size of our mapping populations may limit our ability to detect fine-scale changes.

Linkage relationships: Reconstruction of linkage relationships was straightforward for most linkage groups; homologous segments were supported by numerous markers in the same linear order and most incongruities could be accounted for simple translocations and inversions (*e.g.*, Figure 2). Nonetheless, in some instances individual markers were seemingly misplaced (*i.e.*, found in the wrong position on the right linkage groups). We assumed that most of these minor discrepancies were due to mapping error since it can be difficult to order tightly linked markers, particularly when mapping populations are small. Also, the integration

of multiple maps may introduce inconsistencies in marker order (Burke *et al.* 2004). It is also possible that some of the apparently misplaced markers represent paralogs rather than orthologs.

Because minor differences in ordering were not uncommon, confident identification of inversions is more difficult than translocations. The three inversions recognized in this study (LG12, LG13, and LG16B; supplementary Figures S4 and S5 at http://www.genetics.org/supplemental/) were previously described by Rieseberg *et al.* (1995) and cause reduced introgression rates in natural hybrid zones (Rieseberg *et al.* 1999), so they are likely to be real. However, there was not a sufficient density of informative markers in *H. deserticola* and *H. paradoxus* to test for the presence of the inversions in these species. It is also possible that there have been multiple inversions involving LG17 because marker orders are heterogeneous across species, and this variation in marker order cannot be accounted for by a single inversion. An alternative explanation is that LG17 contains duplicated regions and the proportion of paralagous markers is unusually high.

Despite taking a conservative approach to recognizing chromosomal rearrangements, comparisons of marker locations and ordering revealed that only four linkage groups were collinear across the five species (LG01, LG07, LG09, LG10). The remaining 13 linkage groups were rearranged in one or more species (Figures 2 and 3). The two parental species, *H. annuus* and *H. petiolaris*, differ by at least 11 separate rearrangements, including eight translocations and three inversions (Rieseberg *et al.* 1995; Burke *et al.* 2004). Thus, only 6 of the 17 linkage groups were completely collinear in this comparison.

The genomes of the three hybrid species are extensively rearranged relative to the parental species (Figure 2; Figures S1–S5 available at http://www.genetics.org/supplemental/). *H. anomalus* has the same linkage arrangement as both parents for six linkage groups (LG01, LG03, LG07, LG09, LG10, LG11) and as one or the other parent for an additional three linkage groups (LG05, LG06, LG12B). Note that *H. anomalus* has the same gene order as *H. petiolaris* for inverted regions in LG12 and LG13, while it has the gene order of *H. annuus* for the inversion in LG16B. The eight remaining linkage groups have a novel gene order in *H. anomalus*, and 12 chromosomal breakages/fusions are required to achieve the *H. anomalus* genome from its parental species. While these results are mostly consistent with Rieseberg *et al.* (1995), rearrangements were detected in seven rather than eight linkage groups in the previous study. Specifically, the microsatellite maps reported here imply that the fusion of LG02 and LG08 (L and M in Rieseberg *et al.* 1995) occurred independently in *H. anomalus*; previously, the fused linkage group was assumed to have been derived intact from *H. petiolaris*.

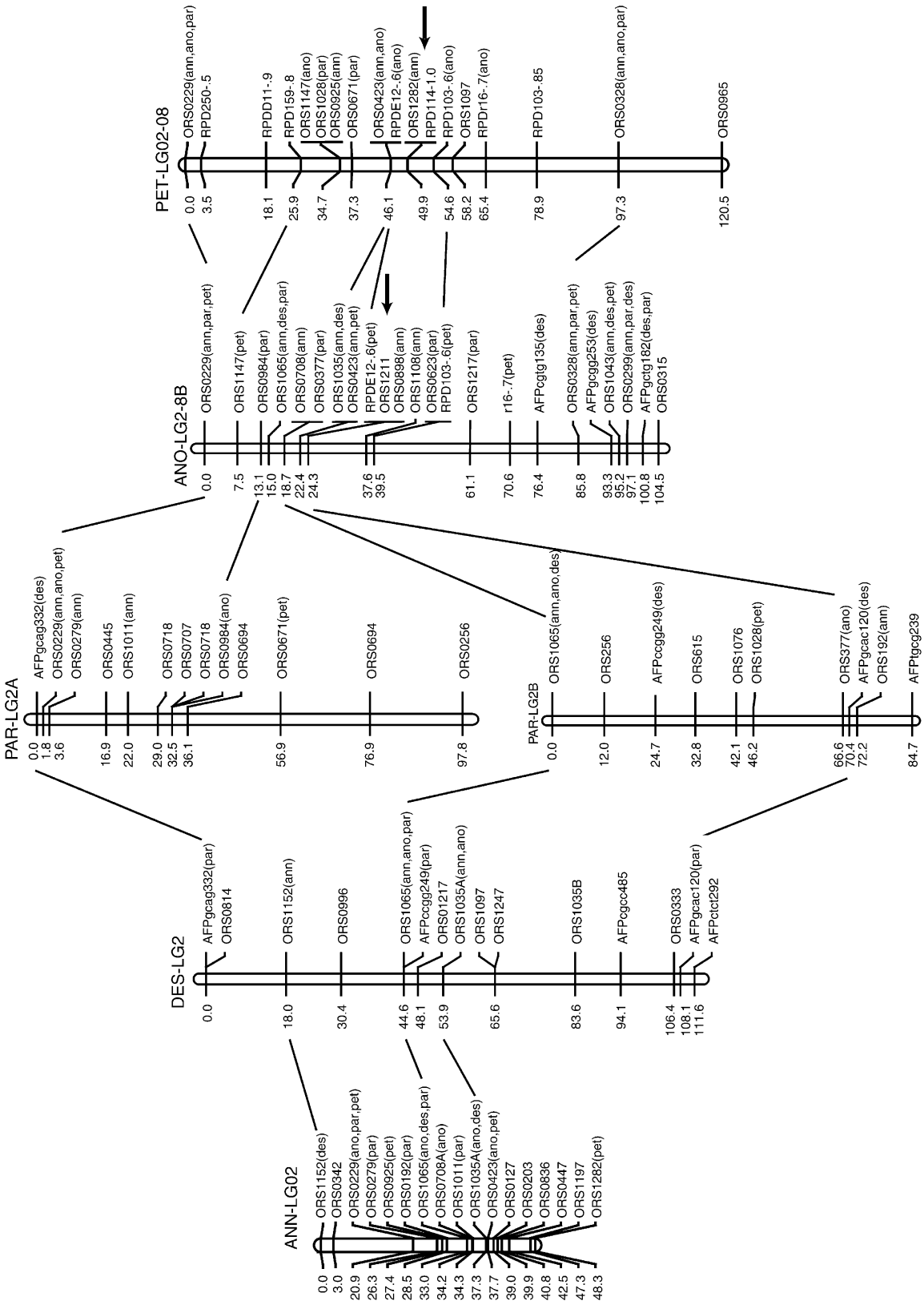
The same general pattern is found in *H. deserticola* (Figure 2; Figures S2, S4, and S5 at http://www.genetics.org/supplemental/). The species has the same linkage arrangement as both parental species for six linkages (LG01, LG03, LG04, LG07, LG09, and LG10), as *H. annuus* for an additional three linkage groups (LG02, LG05, and LG12), and as *H. petiolaris* for two linkage groups (LG06/15 and LG16C). *H. deserticola* has a novel gene arrangement for six linkage groups, which can be accounted for by a minimum of four chromosomal breakages and three fusions relative to the parental species.

Likewise, *H. paradoxus* is collinear with both parental species for six linkage groups (LG01, LG04, LG05, LG07, LG09, and LG10). It resembles *H. annuus* for an additional three linkage groups (LG05, LG08, and LG15) and *H. petiolaris* for one (LG17B). The remaining seven linkage groups in *H. paradoxus* are rearranged relative to both parental species and 10 breakages/fusions are required to account for the evolution of these differences.

Most of the new chromosomal breakages/fusions in the hybrid species (24 of 29) are associated with linkage groups that were already rearranged in the parental species. This is more than expected by chance, even after correcting for the smaller number of collinear than rearranged chromosomes in the parental species ($\chi^2 = 6.86$; $P = 0.009$; note that fusions involving both rearranged and collinear chromosomes were counted as 0.5 in both categories). Also, while the new rearrangements often involve the same linkage groups (particularly LG13, LG14, and LG16), the resulting chromosomal combinations are mostly novel (Figure 2). As a consequence, the hybrid species are as different from each other in chromosome structure as they are from the parental species (Table 2).

QTL analyses of fertility: Eleven QTL were detected for percentage viable pollen in a BC_2 population of the parental species (Table 3). QTL effects were modest in magnitude when estimated in terms of percentage variance explained (PVE), averaging 9.2% (range 5–26%). However, analyses of additive effects suggest a somewhat different story, with an average reduction in the proportion of viable pollen of 19.5% (range 13–27%). This seeming discrepancy is because individuals with four or more sterility QTL often had no viable pollen (*i.e.*, variance in pollen viability has a lower bound of zero), which appears to have resulted in an underestimate of PVE and possibly of additive effects. With respect to the latter, it is noteworthy that individuals with a single sterility QTL had a reduction in pollen viability of as much as 40%. In all instances, the *H. annuus* allele reduced pollen viability in the *H. petiolaris* genetic background.

Nine of the 11 pollen viability QTL map to rearranged linkage groups (Table 3), which is significantly more than expected by chance ($\chi^2 = 3.97$; $P = 0.046$), despite the preponderance of rearranged linkage groups (11 rearranged *vs.* 7 collinear) differentiating the parental species. Of the 9 QTL located on rearranged linkage groups, 8 map close to chromosomal breakpoints,


PET-LG02-08
0.0 ORS0229(ann,ano,par)
3.5 RPD250-.5
18.1 RPDD11-.9
25.9 RPD159-.8
34.7 ORS1147(ano)
37.3 ORS1028(par)
46.1 ORS0925(ann)
49.9 ORS0671(par)
54.6 ORS0423(ann,ano)
58.2 RPDE12-.6(ano)
65.4 ORS1282(ann)
78.9 RPD114-1.0
97.3 RPD103-.6(ano)
120.5 ORS1097
RPDr16-.7(ano)
RPD103-.85
ORS0328(ann,ano,par)
ORS0965
ANO-LG2-8B
0.0 ORS0229(ann,par,pet)
7.5 ORS1147(pet)
13.1 ORS0984(par)
15.0 ORS1065(ann,des,par)
18.7 ORS0708(ann)
22.4 ORS0377(par)
24.3 ORS1035(ann,des)
ORS0423(ann,pet)
37.6 RPDE12-.6(pet)
39.5 ORS1211
ORS0898(ann)
ORS1108(ann)
61.1 ORS0623(par)
RPD103-.6(pet)
ORS1217(par)
70.6 r16-.7(pet)
76.4 AFPcgtg135(des)
85.8 ORS0328(ann,par,pet)
93.3 AFPgcgg253(des)
95.2 ORS1043(ann,des,pet)
97.1 ORS0299(ann,par,des)
100.8 AFPgctg182(des,par)
104.5 ORS0315
PAR-LG2A
0.0 AFPgcag332(des)
1.8 ORS0229(ann,ano,pet)
3.6 ORS0279(ann)
16.9 ORS0445
22.0 ORS1011(ann)
29.0 ORS0718
32.5 ORS0707
36.1 ORS0718
ORS0984(ano)
ORS0694
56.9 ORS0671(pet)
76.9 ORS0694
97.8 ORS0256
PAR-LG2B
0.0 ORS1065(ann,ano,des)
12.0 ORS256
24.7 AFPccgg249(des)
32.8 ORS615
42.1 ORS1076
46.2 ORS1028(pet)
66.6 ORS377(ano)
70.4 AFPgcac120(des)
72.2 ORS192(ann)
84.7 AFPtgcg239
DES-LG2
0.0 AFPgcag332(par)
ORS0814
18.0 ORS1152(ann)
30.4 ORS0996
44.6 ORS1065(ann,ano,par)
48.1 AFPccgg249(par)
53.9 ORS01217
ORS1035A(ann,ano)
65.6 ORS1097
ORS1247
83.6 ORS1035B
94.1 AFPcgcc485
106.4 ORS0333
108.1 AFPgcac120(par)
111.6 AFPctct292
ANN-LG02
0.0 ORS1152(des)
3.0 ORS0342
20.9 ORS0229(ano,par,pet)
26.3 ORS0279(par)
27.4 ORS0925(pet)
28.5 ORS0192(par)
33.0 ORS1065(ano,des,par)
34.2 ORS0708A(ano)
34.3 ORS1011(par)
37.3 ORS1035A(ano,des)
37.7 ORS0423(ano,pet)
39.0 ORS0127
39.9 ORS0203
40.8 ORS0836
42.5 ORS0447
47.3 ORS1197
48.3 ORS1282(pet)

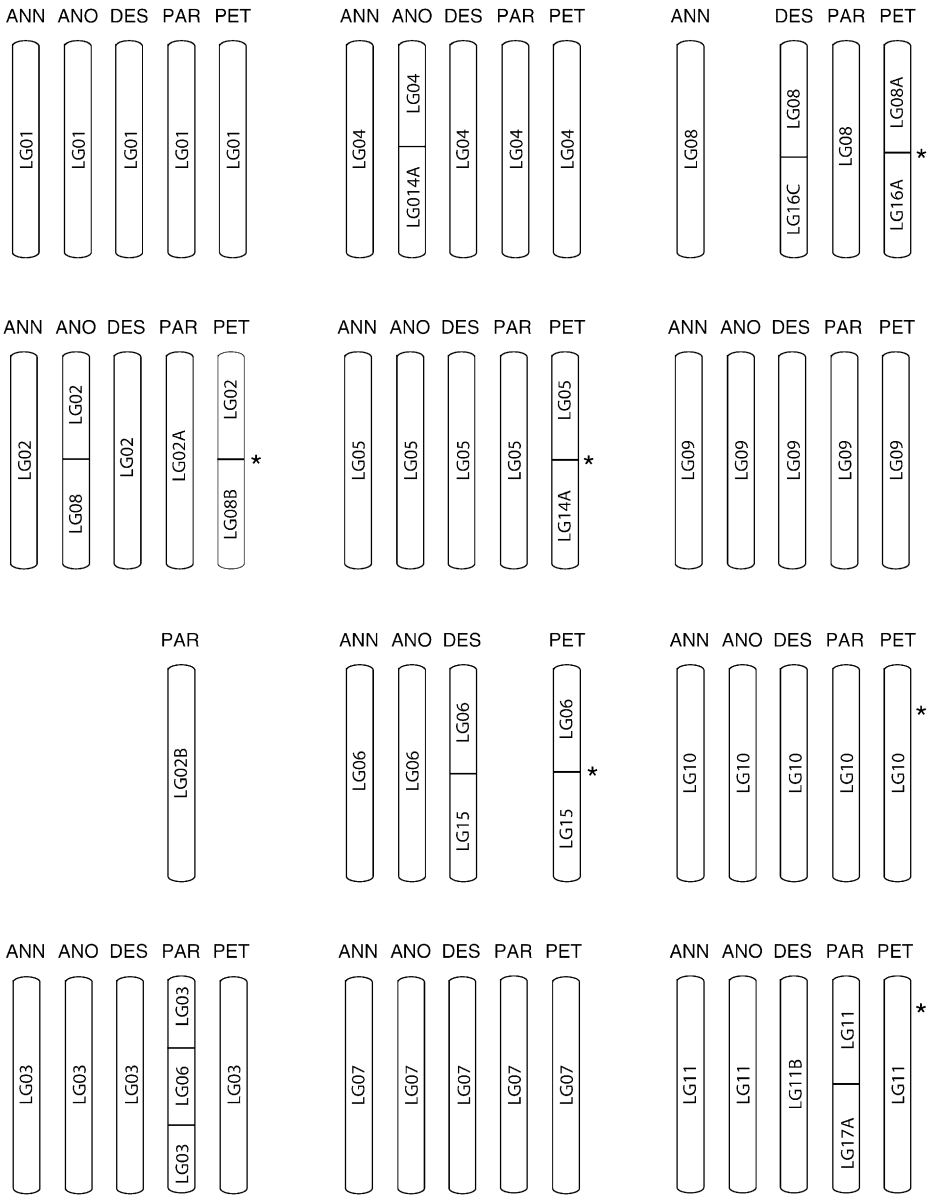


FIGURE 3.—Inferred chromosomal structural relationships between the parental species, *H. annuus* and *H. petiolaris*, and their three diploid hybrid derivatives, *H. anomalus*, *H. deserticola*, and *H. paradoxus*. Segments containing inversions are indicated by hatched lines. Note that there was insufficient marker density in *H. deserticola* and *H. paradoxus* to evaluate the presence or absence of inversions. Asterisks indicate the approximate position of pollen viability QTL (*cf.* Table 3).

implying that the rearrangement itself might be responsible for the observed reduction in fertility. While this observation is consistent with previous reports of complex multivalent formation in the meiosis of sunflower hybrids, it cannot be proven because gene incompatibilities may also cluster near chromosomal breakages (NOOR *et al.* 2001; NAVARRO and BARTON 2003). The two QTL not associated with rearranged linkage groups map to genomic regions that were previously shown to be associated with reductions in pollen viability and reduced gene flow in natural hybrid zones of *H. annuus* and *H. petiolaris* (RIESEBERG *et al.* 1999).

Epistasis: The genome-wide scan of digenic interactions detected 13 significant interactions (Table 4).

FIGURE 2.—Gene order relationships for a representative linkage group (LG2) between two parental sunflower species, *H. annuus* and *H. petiolaris*, and their three diploid hybrid derivatives, *H. anomalus*, *H. deserticola*, and *H. paradoxus*. Note the fission of LG2 in *H. paradoxus* and the fusion of linkages 2 and 8 in *H. anomalus* and *H. petiolaris*. Markers beginning with ORS are simple sequence repeats (SSRs), those beginning with AFP are AFLPs, and those beginning with RPD are RAPDs. Markers followed by species names in parentheses (ann, *annuus*; ano, *anomalus*; par, *paradoxus*; and pet, *petiolaris*) are informative and map to the same linkage group in the listed species. Informative loci on adjacent linkage groups are also connected by lines. Numbers to the left of each linkage group refer to genetic distance (centimorgans). Single arrows indicate the location of inferred chromosomal breakages/fusions that are necessary to account for the differences between this map and that of *H. annuus* (Figure S4 at http://www.genetics.org/supplemental/). Letters (A and B) following some linkage group designations indicate fragmented linkage groups, with fragments homologous to the top of the *H. annuus* linkage group designated A, the next highest fragment labeled B, and so forth.

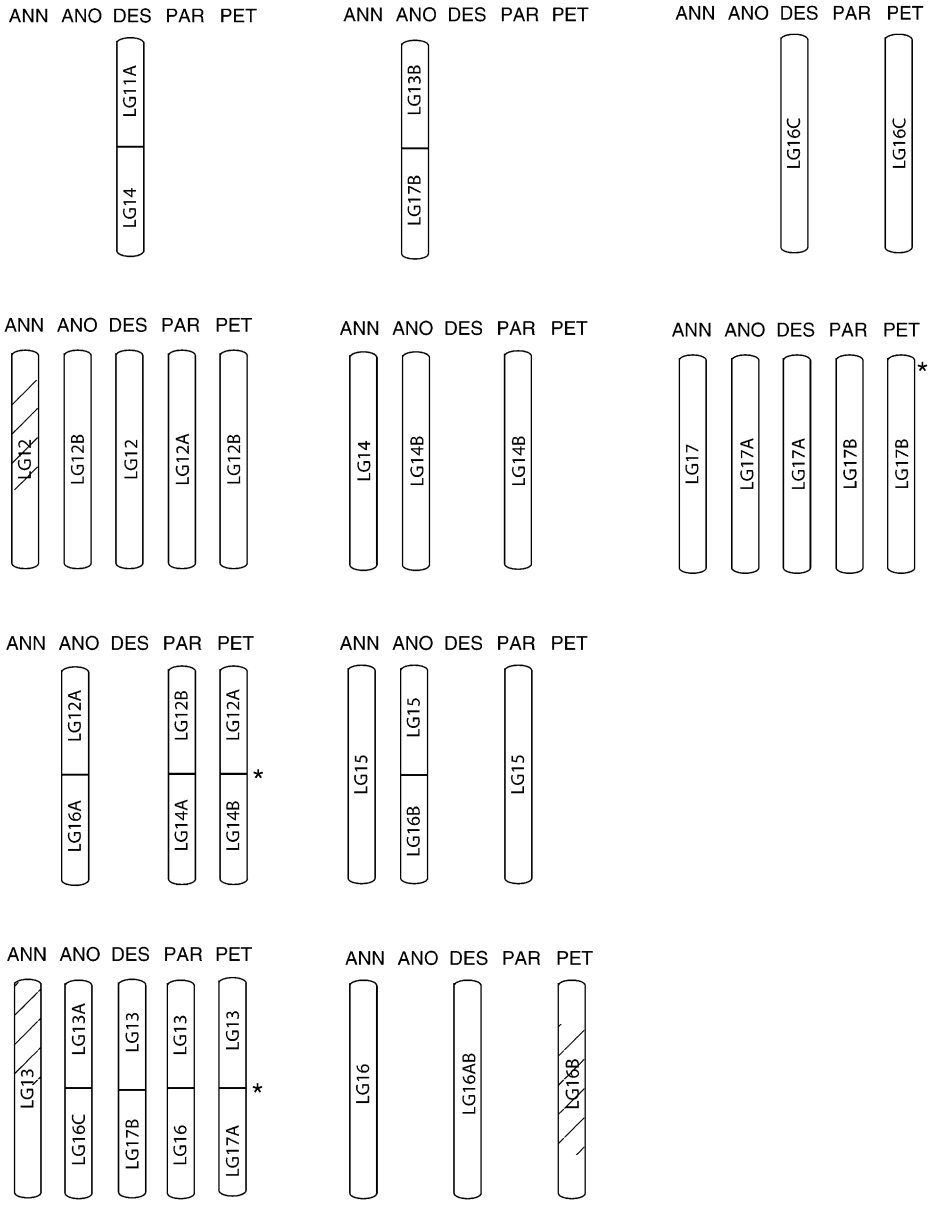


FIGURE 3.—*Continued.*

However, many of the interactions involved linked markers from the same pair of chromosomes and may represent as few as 6 independent interactions. The clearly independent interactions involve six previously detected QTL from rearranged linkage groups (Table 3), as well as two new fertility QTL from collinear linkages (LG1 and LG3). Although multivalent formation during meiosis could generate nonadditive effects

TABLE 2

Percentage pollen viability (on and above the diagonal) and the number of rearranged chromosomes (below diagonal) derived from crosses within and among populations of *H. annuus*, *H. petiolaris*, and their stabilized hybrid derivatives, *H. anomalus*, *H. deserticola*, and *H. paradoxus*

Taxon	*H. annuus*	*H. anomalus*	*H. deserticola*	*H. paradoxus*	*H. petiolaris*
H. annuus	94.3 (0.7)	2.6 (0.1)	4.9 (0.9)	9.1 (0.5)	4.8 (0.2)
H. anomalus	9	94.5 (0.9)	42.9 (3.9)	14.8 (2.8)	3.0 (0.1)
H. deserticola	9	10	90.3 (3.1)	35.9 (3.9)	2.5 (0.7)
H. paradoxus	9	11	11	95.6 (1.1)	10.9 (0.6)
H. petiolaris	10	10	9	11	94.9 (0.8)

Standard errors are in parentheses. Pollen viabilities for crosses among *H. annuus*, *H. anomalus*, and *H. petiolaris* are from RIESEBERG (2000).

TABLE 3

Putative QTL positions, likelihood ratios (LR), percentage variance explained (PVE), additive effects, and significance levels for the percentage viable pollen in a BC_2 population of the wild sunflower species *H. annuus* × *H. petiolaris*

Linkage group[a]	Position (cM)	1-LOD interval	Interval markers	LR	PVE (%)	Additive effect	*P*
2[b]	49	47-49	ORS203	109.3	26	−0.27	< 0.005
5[b]	1	0-4	ORS547-ORS240	28.2	6	−0.18	< 0.005
6a[b]	3	0-8	ac/ctg281-ta/gc262	24.2	5	−0.23	< 0.005
8a[b]	44	39-44	ac/tg124-ac/ctg206	54.3	10	−0.17	< 0.005
10	11	5-14	ORS256	22.1	7	−0.17	< 0.005
11	48	45-49	ORS1146-ORS261	18.2	6	−0.14	< 0.05
12[b]	7	0-15	ORS963-ORS984	14.7	12	−0.23	< 0.05
14[b]	62	57-64	ac/ctg189	27.5	5	−0.13	< 0.005
16[b]	5	0-10	ORS1017-ac/ctg196	27.4	5	−0.24	< 0.005
17a	45	39-52	ORS1097-ORS1187	67.1	14	−0.25	< 0.005
17b[b]	0	0-14	ORS727-ORS845	25.0	5	−0.13	< 0.005

A description of the mapping population, molecular markers employed, and map positions may be found in Lexer *et al.* (2003a) and Rieseberg *et al.* (2003).

[a] Lowercase letters following linkage group numbers refer to fragmented segments in the Rieseberg *et al.* (2003) population and do not correspond to the uppercase letters employed in Figure 2 and Figures S1–S5 (available at http://www.genetics.org/supplemental/).

[b] Pollen viability QTL maps near rearrangement breakpoint.

on fertility (Gardner *et al.* 2000), the epistasis detected here appears to be the result of interactions among genes since none of the interactions involve linkage groups that would be part of the same multivalent configuration. Also, two of the epistatic QTL derive from collinear linkages and were detected on the basis of their interaction effects rather than their additive effects.

Crossing relationships: First-generation hybrids derived from all pairwise combinations of crosses (Table 2; Figure 4) reveal that the three hybrid species are strongly isolated from their parents. Percentages of viable pollen range from 2.5 ± 0.7% for crosses between *H. deserticola* and *H. petiolaris* to 10.9 ± 0.6% for crosses between *H. paradoxus* and *H. petiolaris* (Table 2), whereas percentage seed set (Figure 4) is lowest in crosses between *H. anomalus* and *H. annuus* (0.16 ± 0.04%) and highest for crosses between *H. deserticola* and *H. petiolaris* (2.2 ± 0.7%).

The hybrid species are isolated from each other as well, but the sterility barrier is not as strong, with the pollen fertility of first-generation hybrids ranging between 14.8 ± 2.8% for crosses between *H. anomalus* and *H. paradoxus* and 42.9 ± 3.9% for crosses between

TABLE 4

Significant digenic interactions ($P \leq 1 \times 10^{-5}$) and likelihood ratios (LR) for percentage of viable pollen in a BC_2 population of *H. annuus* × *H. petiolaris*

Independent interactions	Locus A	Locus B	LR total effect	LR interaction effect
1	ORS371(1)	ORS826(8a)	27.3	6.7
1	ORS371(1)	ac/ctg206(8a)	51.4	8.8
2	ORS447(2)	ORS1114(3)	26.7	7.6
3	ORS447(2)	ORS1187(17a)	47.0	9.3
3	ccgg249 (2)	ORS1187(17a)	77.5	8.2
3	ORS708(2)	ORS1187(17a)	73.4	9.0
3	ORS203(2)	ORS847(17a)	105.3	7.2
4	ORS1114(3)	ORS1108(8a)	37.9	7.0
4	ORS1114(3)	ORS826(8a)	35.7	6.7
4	ORS1114(3)	ac/ctg206(8a)	59.7	7.1
5	ORS1108(8a)	ac/ctg189(14)	49.2	9.8
5	ORS826(8a)	ac/ctg189(14)	46.0	8.9
6	ORS963(12)	ORS727(17b)	52.6	6.8

A description of the mapping population, molecular markers employed, and map positions may be found in Lexer *et al.* (2003a) and Rieseberg *et al.* (2003). Linkage group is given in parentheses following locus name.

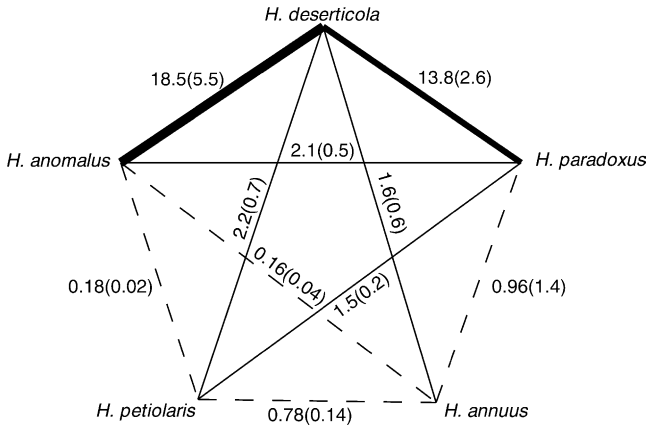


FIGURE 4.—Percentage seed set of first-generation hybrids between the parental species, *H. annuus* and *H. petiolaris*, and their three diploid hybrid derivatives, *H. anomalus*, *H. deserticola*, and *H. paradoxus*. Values are presented as mean (standard error). Line thickness is proportional to cross-compatibility. Dashed lines indicate that some individuals were completely sterile for that cross. Values for crosses among *H. annuus*, *H. petiolaris*, and *H. anomalus* derive from RIESEBERG (2000).

H. anomalus and *H. deserticola*. Analyses of percentage seed set provides a similar estimate of barrier strength, with first-generation hybrids between *H. anomalus* and *H. paradoxus* producing only 2.1 ± 0.5% viable seed compared to 18.5 ± 5.5% viable seed for crosses between *H. anomalus* and *H. deserticola*.

In contrast to the interspecific crosses, all intraspecific crosses were fully fertile, with the percentage of viable pollen consistently >90% (Table 2) and the percentage seed set >80% (not shown). Lower fertility has been previously reported for some cross-combinations with *H. anomalus* and *H. deserticola* (SCHWARZBACH and RIESEBERG 2002; GROSS *et al.* 2003), but these involved populations that appear to have been independently derived (*H. anomalus* and *H. deserticola* appear to have multiple origins). The intraspecific crosses reported here involve populations from the same origin.

Pollen and seed fertility are highly correlated across crosses ($r = 0.97$; $P \ll 0.001$), but the direction of the cross did not have a significant effect on fertility in any of the 10 cross-combinations. Thus, interactions between the cytoplasmic and nuclear genes do not appear to be important contributors to the evolution of sterility barriers between these sunflower species. This differs from recent reports emphasizing the ubiquity of asymmetric reproductive barriers in angiosperms (TIFFIN *et al.* 2001; LEVIN 2003). Also, there was not a significant correlation between the total number of rearrangements that differentiate the species and pollen ($r = 0.49$; $P = 0.09$) or seed fertility ($r = 0.33$; $P = 0.21$). However, this should not be viewed as evidence that chromosomal rearrangements do not affect fertility, because there is very little variance in the number of rearranged chromosomes differentiating the species (Table 2). Nonetheless, the fact that the hybrid species differ by a similar number of rearranged chromosomes as do the parental species, yet have weaker sterility barriers, does imply that genic incompatibilities are significant contributors to sterility as well. This argument is further buttressed by the fact that the two hybrid species that are most similar in gene composition, *H. anomalus* and *H. deserticola* (RIESEBERG *et al.* 2003), are also most interfertile (Table 2; Figure 4), despite differing by 10 rearranged chromosomes.

DISCUSSION

Homoploid hybrid speciation: For new homoploid hybrid species to become established, they must somehow become reproductively isolated from their parental species. This is a difficult step. Unlike allopolyploidy where genome doubling provides instantaneous isolation, there is no simple means by which a homoploid hybrid can become isolated. Verbal models suggest that a hybrid neospecies might become isolated through rapid karyotypic evolution (*i.e.*, the recombinational speciation model; STEBBINS 1957; GRANT 1981), ecological divergence (GRANT 1981; TEMPLETON 1981), and/or spatial isolation, perhaps mediated by hybrid founder events (CHARLESWORTH 1995). Simulation models confirmed the feasibility of these various scenarios (MCCARTHY *et al.* 1995; BUERKLE *et al.* 2000), but show that while ecological divergence alone was sufficient for a new hybrid species to arise, it was unlikely to evolve independently without significant karyotypic change and/or spatial isolation. Thus, an important empirical question is whether rapid karyotypic evolution often accompanies the origin of homoploid hybrid species.

This study provides important confirmation of the recombinational speciation model, at least in Helianthus. The three hybrid sunflower species differ from their parents by a minimum of nine rearranged chromosomes (Table 2; Figure 3). About one-third of the differences in karyotype have arisen from the simple sorting of chromosomal rearrangements that differentiate the parental species, whereas the remainder have arisen *de novo* (six breakages/six fusions in *H. anomalus*; four breakages/three fusions in *H. deserticola*, and five breakages/five fusions in *H. paradoxus*). The fact that a significant overproportion of new rearrangements involved linkages that were already rearranged in the parental species, as opposed to those that are collinear, implies that chromosomal breakage in hybrids may be a consequence of meiotic abnormalities involving rearranged linkage groups (TEMPLETON 1981; RIESEBERG *et al.* 1995). However, it is also possible that these linkage groups are susceptible to chromosomal mutation for other reasons.

Despite the consistent association between chromosomal repatterning and homoploid hybrid speciation

in Helianthus, chromosomal change is not universally associated with this mode of speciation. Of ~11 confirmed examples of homoploid hybrid speciation (Gross and Rieseberg 2005), karyotypic change has been reported in six cases—the three Helianthus examples plus *Argyranthemum sundingii* (Brochmann *et al.* 2000; Borgen *et al.* 2003), *Iris nelsonii* (Randolph 1966; Arnold 1993), and *Stephanomeria diegensis* (Gallez and Gottlieb 1982). Also, some karyotypic races of house mice are speculated to have arisen through hybridization (Pialek *et al.* 2001). However, only in Helianthus has there been detailed analyses of the hybrid species' genomes.

Even less is known about karyotypic evolution in the other five homoploid hybrid taxa: *Daphnia mendota* (Taylor *et al.* 1996), *Gila seminuda* (Demarais *et al.* 1992), Paeonia spp. (Sang *et al.* 1995,1997; Sang and Zhang 1999), *Penstemon clevelandii* (Wolfe *et al.* 1998), and *Pinus densata* (Wang *et al.* 2001). Habitat and/or pollinator isolation is considered to be the predominant form of isolation in each case, but as far as we are aware the only formal karyotypic analysis has been conducted in Pinus (Yang 1987); *P. densata* was shown to have the same karyotype as one of its parental species (Yang 1987; Wang *et al.* 2001).

Chromosomal rearrangements and sterility: An underlying assumption of the recombinational model that has not previously been verified is that the karyotypic changes accompanying speciation are underdominant and create a sterility barrier with the parental species. The QTL analyses reported here indicate that pollen sterility QTL in Helianthus are significantly more likely to occur on rearranged than on collinear linkage groups and that sterility QTL in rearranged chromosomes almost always map near chromosomal breakpoints. Chromosomal rearrangements in Helianthus have previously been shown to generate multivalent configurations in meiois (Heiser 1947; Chandler *et al.* 1986). Thus, the rearrangements themselves appear to be at least partially responsible for the fertility reductions observed in hybrids.

Interspecific incompatibilities among genes also contribute to hybrid sterility in Helianthus. The best evidence for this is that, as previously reported by Rieseberg *et al.* (1999), several sterility QTL map to collinear rather than to rearranged linkage groups. Also, because none of the interactions involve linkage groups that would be part of the same multivalent configuration, the epistasis detected here is likely to be the result of interactions among genes. This is consistent with theory and empirical evidence, which indicate that genic incompatibilities are likely to cluster within or near chromosomal rearrangements (Noor *et al.* 2001; Navarro and Barton 2003; Brown *et al.* 2004). Finally, crosses among the hybrid species, which are similar in gene composition (Rieseberg *et al.* 2003), but not in karyotype, were more fertile than crosses between the parental species, despite differing by similar numbers of chromosomal rearrangements.

It is difficult to evaluate the relative contributions of genic incompatibilities *vs.* chromosomal rearrangements to hybrid sterility. A crude estimate can be gleaned by analyzing the genomic distribution of sterility QTL; nine of 11 sterility QTL map to rearranged linkage groups and account for 87% of the phenotypic variance for pollen sterility. However, at least some of the sterility mapping to rearranged linkage groups must be caused by genes. If we assume an even distribution of sterility QTL across the genome, then ~35% of the reduction in pollen viability may be attributed to genes and 65% to chromosomal rearrangements. Even this is likely to be an underestimate of the contribution of genes to reduced pollen viability, because genic incompatibilities are likely to be recessive (Orr and Presgraves 2000; Fishman and Willis 2001), epistatic effects on sterility are not considered, and genic incompatibilities may accumulate disproportionately on rearranged chromosomes (Noor *et al.* 2001).

Reproductive barrier strength and the independent evolution of hybrid neospecies: Computer simulation models indicate that in the absence of a strong sterility barrier, a new hybrid lineage is unlikely to evolve independently from its parental species (Buerkle *et al.* 2000). Instead, the hybrid population is likely to represent a steep step in a cline between the parental populations. Evolutionary independence can be achieved if there are strong ecological and sterility barriers (*i.e.*, hybrid fertility < 0.1). This requirement does appear to be met in Helianthus. All pollen and seed fertility estimates for F_1's between the hybrid species and their parents fall below 11%, and we have previously documented the occurrence of strong ecological selection in the habitats of all three hybrid species (Lexer *et al.* 2003a,b; Gross *et al.* 2004; Ludwig *et al.* 2004).

In conclusion, the requirements of the recombinational speciation model do appear to be fulfilled in Helianthus. The hybrid species are strongly divergent karytoypically, the karyotypic differences contribute to reproductive isolation, and barrier strength is sufficient to achieve evolutionary independence in parapatry. However, it remains to be determined whether homoploid hybrid species in other groups of plants and animals satisfy the recombinational mode. It might be, for example, that some of the more weakly isolated hybrid taxa will exhibit little genetic isolation from their parental species (*i.e.*, only slightly reduced gene flow) when tested and are better viewed as introgresssive races. More generally, there is a need to discriminate between the effects of chromosomal rearrangements and linked genic incompatibilities in both plants and animals. This seemingly requires a laborious combination of fine-mapping and map-based cloning, although some inferences may be made by studying patterns of interfertility in different hybrid classes (*e.g.*, Fishman and Willis 2001).

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