

FIG. 3 Relationship between experimental manipulation (change in brood size) and forehead patch size ($X \pm s.e.$) of first-year males recruited from experimental nests (mean per nest). The relationship is significant (ordered heterogeneity test²², $r_{SPc} = 0.663$, $k = 5$, $P < 0.02$). Patch size in males was measured at the time when they were feeding nestlings in their first year. Numbers above bars show number of males. The analysis is restricted to experiments done in 1983–85 as brood-size manipulations were not independent of clutch size in 1989–90; there were no indications of year effects on patch size of recruited males (multiple regression with years as dummy variables, $P > 0.47$).

and ornamentation², as do some comparative studies of mortality in relation to sexual dimorphism²⁰, but this is the first direct evidence for a trade-off. The relationship between patch size and polygyny found in this study suggests that increased parental effort may reduce male attractiveness in subsequent years, by reducing the size of a condition-dependent secondary sexual character. Because increased parental effort by males may reduce sexually selected components of reproductive success, models of parental investment²¹ or sexual ornamentation⁸ must consider the trade-off between the two. □

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Hybrid speciation accompanied by genomic reorganization in wild sunflowers

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THE origin of a new diploid species via hybridization is theoretically difficult because it requires the development of reproductive isolation in sympatry. In the absence of isolation, the hybrid genotype will be overcome by gene flow with its parents. According to genetic models^{1–3}, reproductive isolation can be facilitated by rapid karyotypic evolution in the recombinant hybrid. Here we use comparative linkage mapping^{4,5} to demonstrate extensive genomic reorganization in the hybrid species *Helianthus anomalus*, relative to its parents *H. annuus* and *H. petiolaris*. The unprecedented detail provided by the linkage maps indicates that rapid karyotypic evolution in *H. anomalus* results from the merger of pre-existing structural differences between the parents, as well as chromosomal rearrangements apparently induced by recombination. Moreover, determination of the parental origin of mapped loci in *H. anomalus* suggests that parental genomic structure has influenced hybrid genomic composition by protecting several large linkage blocks from recombination during speciation. These mapping data, when combined with previous meiotic analyses⁶ and evidence of semisterility between the hybrid and its parents^{6,7}, satisfy genetic models for speciation through hybrid recombination.

The three species under investigation are self-incompatible annual sunflowers with the same chromosome number ($n = 17$)⁸. The parents, *Helianthus annuus* and *H. petiolaris*, are widespread polytypic species that are distinguished by several morphological and chromosomal features⁸, occur in divergent clades based on chloroplast DNA⁹ and nuclear ribosomal DNA¹⁰ variation, and have different ecological requirements. In general, *H. annuus* is restricted to heavy, clay soils and *H. petiolaris* to dry, sandy soils. Nevertheless, the two species are sympatric throughout much of western USA, and hybrid swarms are common. First-generation hybrids are semisterile, with pollen viabilities typically less than 10% and seed set less than 1% (refs 6 and 11), whereas F₂ pollen viability is highly variable, ranging from 13% to 97% (ref. 11).

By contrast, the hybrid *H. anomalus* is a rare species endemic to xeric habitats in northern Arizona and southern Utah^{7,12}, well within the range of its parents. Although *H. anomalus* is morphologically distinct from *H. annuus* and *H. petiolaris*, it combines their ribosomal DNA repeat units¹⁰ and allozymes⁹, as predicted for a diploid hybrid species¹³. In addition, individuals of the species possess the chloroplast DNA haplotype of *H. annuus* or *H. petiolaris* rather than a unique one⁹.

We generated genetic linkage maps for all three species by using random amplified polymorphic DNA (RAPD)¹⁴ markers (Fig. 1; see also Supplementary Information). To map dominant markers, intraspecific hybrids were generated between geographically isolated populations of each species and then crossed to an inbred sunflower line (CMS89). Mapping progenies of 50 to 60 individuals were grown to a size sufficient for total DNA isolations¹⁵, and selected individuals were surveyed for genetic polymorphisms shared by at least two mapping populations. From a survey of 715 single primers or primer pairs, 174 single primers and 13 primer pairs were chosen for mapping. For each mapping population, all segregating loci were scored, and linkage maps were generated with the computer program MAP-MAKER (ref. 16). Loci were distributed onto 17 linkage groups in each map, corresponding to the haploid chromosome number

of the three species. Because of differences in levels of polymorphism among mapping populations, the number of loci mapped varied from 212 in *H. annuus* to 400 in *H. petiolaris* (Fig. 1; see also Supplementary Information). Total map length also varied by a factor of two (Fig. 1; also Supplementary Infor-

mation), but map density was similar among the three species, with an average distance between loci ranging from 4.6 cM in *H. petiolaris* to 6.2 cM in *H. anomalous*.

By comparing the genomic location and linear order of homologous markers, we inferred chromosomal structural rela-

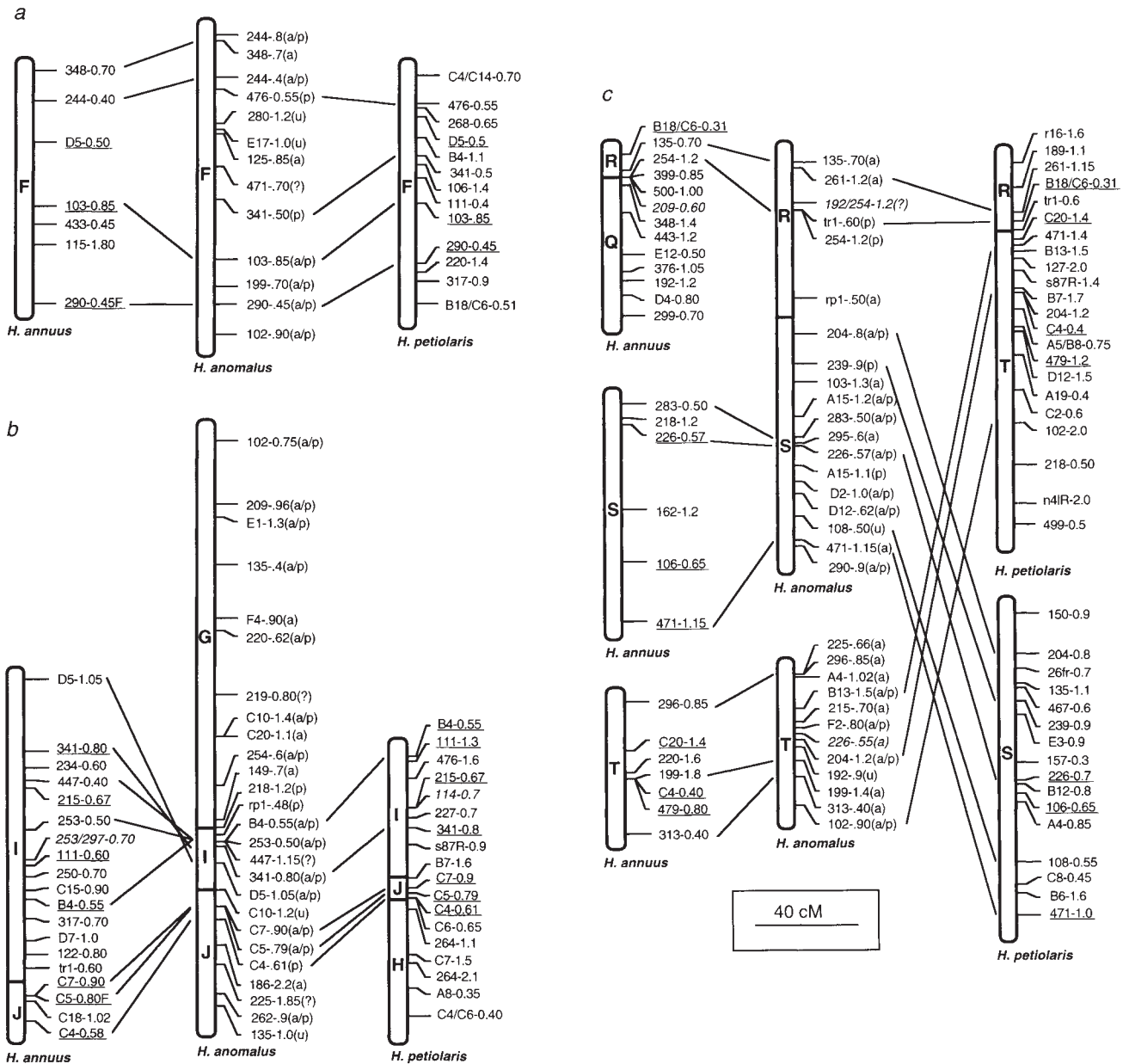


FIG. 1 Selected linkages from the two parental species, *H. annuus* and *H. petiolaris*, and their hybrid, *H. anomalous*, which illustrate the three types of structural relationships observed among the linkage maps: a, complete collinearity; b, inversions; and c, interchromosomal translocations. Letters within each linkage group designate major linkage blocks and indicate their relationship to homologous linkages in the other species. Locus nomenclature includes, from left to right, the primer designation and the size in kilobases of the segregating fragment scored. For the *H. anomalous* linkages, the letters in parentheses following each locus/allele indicate its parental origin: a, *H. annuus*; p, *H. petiolaris*; a/p, *H. annuus* or *H. petiolaris*; u, unique to *H. anomalous*; and ?, data not available. Lines between linkages indicate homologous loci between the hybrid species and its parents, whereas homologous loci between the two parental species are underlined. Loci italicized also show homology with loci from other mapping populations, but their genomic locations are incongruent with most homologous loci, suggesting paralogous rather than orthologous relationships.

METHODS. Primers 101–500 are from the University of British Columbia Biotechnology Laboratory, and primers A1–A20, B1–B20, C1–C20, D1–D20, E1–E20 and F1–F20 are from Operon Technologies. The remaining primers are 20–30 bases in length, and sequences can be obtained from the authors upon request. In some instances, RAPD primers were paired for amplification. Amplification and electrophoresis with single primers followed standard RAPD protocols¹⁴, whereas for amplification with RAPD primer pairs the extension time was reduced from 2 min to 10 s, and electrophoresis was performed on 2.0% agarose gels. Homology among RAPD fragments generated by the same primer was verified by Southern hybridization¹⁵ or by restricting gel-isolated fragments and observing congruent band profiles²¹. Maps were developed with the computer program MAPMAKER (ref. 16) using a Lod score of 4.0 and a recombination limit of 0.25. Recombination values were converted to map distances using the Kosambi mapping function. Potential scoring and ordering errors were detected using the 'genotype' command of MAPMAKER, and areas with apparent discrepancies were rescored or retested.

tionships among the three species (Figs 1 and 2; also Supplementary Information). Six linkages were collinear among all three species, whereas the remaining 11 linkages were not conserved in terms of gene order. The two parental species, *H. annuus* and *H. petiolaris*, differ by at least ten separate structural rearrangements, including three inversions and a minimum of seven interchromosomal translocations.

The genome of the hybrid, *H. anomalus*, was extensively rearranged relative to its parents (Figs 1 and 2; see also Supplementary Information). For 4 of the 11 rearranged linkages, *H. anomalus* shared the linkage arrangement of one parent or the other. For the remaining 7 linkages, however, unique linkage arrangements were displayed. In fact, a minimum of three chromosomal breakages, three fusions, and one duplication are required to achieve the *H. anomalus* genome from its parents. All seven novel rearrangements in *H. anomalus* involve linkage groups that are structurally divergent in the parental species, suggesting that structural differences may induce additional chromosomal rearrangements upon recombination¹⁷.

In addition to generating genomic maps for each species, we also attempted to determine the parental origin of each locus mapped in *H. anomalus*, by surveying natural populations of each parental species for loci or alleles mapped in *H. anomalus*¹⁵. A preliminary survey of 162 loci¹⁵ revealed that most *H. anomalus* linkage groups had loci derived from both parental species. However, several large blocks of markers appeared to have been transmitted intact from one or the other parental species, and it was speculated that these regions were protected from recombination by structural differences in the parental genomes¹⁵. Parental origin data from all 357 mapped loci (Fig. 1 and Supplementary Information) confirm the integrity of three of these blocks of genes, all of which occur in structurally divergent linkage groups (Figs 1 and 2; also Supplementary Information). All three blocks are derived from one of the parental species, *H. annuus*. A possible mechanism for the preservation of several linkage blocks from a single parent might be epistasis.

To determine whether the blocks of genes preserved in the hybrid are longer (in terms of an unbroken sequence of markers from one parent) than would be expected by chance, simulations of marker distributions within the *H. anomalus* genome were performed. Because markers that were polymorphic in both parents or unique to *H. anomalus* provide no information about recombination, the simulations were restricted to the 157 markers of known parental origin. Thus for each simulation we randomly sampled the length and distribution of blocks of genes within the entire genome, as well as within individual linkage groups or rearranged linkage blocks. The simulations were repeated 1,000 times. Results from the simulations revealed that three unbroken sequences of six, seven and seven or more markers from one parent, such as those observed in *H. anomalus*, would be unlikely to occur by chance alone ($P=0.021$).

To reduce gene flow, chromosomal structural differences must enhance reproductive isolation. This does appear to be the case in *H. anomalus*, where first-generation hybrids with their parents are partially sterile, with pollen stainabilities of 1.8% to 4.1% (*H. annuus*) and 2.0% to 58.4% (*H. petiolaris*)^{6,7}. Meiotic analyses revealed multivalent formations, and bridges and fragments, suggesting that chromosomal structural differences are largely responsible for hybrid semisterility⁶.

Because individual selection cannot explain the evolution of hybrid sterility¹⁸, the accumulation of additional chromosomal rearrangements in the ancestral hybrid population should be viewed as a chance consequence of recombination rather than an adaptive response to selection. Although these structural changes currently serve to reduce gene flow between *H. anomalus* and its parents, their role during the origin of the species is less clear. Theory suggests that the higher the number of structural polymorphisms in the ancestral hybrid population, the lower is the probability of generating new structural homozygotes². Outcrossing, which is typical of *Helianthus*⁸, would also make

fixation more difficult^{2,3}. Selfing can be induced in hybrids of *H. annuus* and *H. petiolaris*¹¹, but its frequency in natural hybrids is unknown. However, structural homozygotes generated from highly polymorphic hybrid populations are more likely to be unique and intersterile with each other and with their parents². Thus the presence of additional structural rearrangements in hybrid populations will reduce the probability of generating fertile hybrid progeny, but if fertile recombinants are produced they are less likely to be swamped out of existence by gene flow.

Genetic models for hybrid recombination¹⁻³ imply ecological divergence as well as karyotypic evolution, because the stabilized hybrid must either coexist with its parents or become adapted to a new habitat or niche³. This model is corroborated in *Helianthus*, as *H. anomalus* shows ecological divergence from both parents, occupying xeric habitats. A similar pattern of chromosomal and ecological divergence has been reported for two other diploid hybrid species in *Helianthus*^{10,19}.

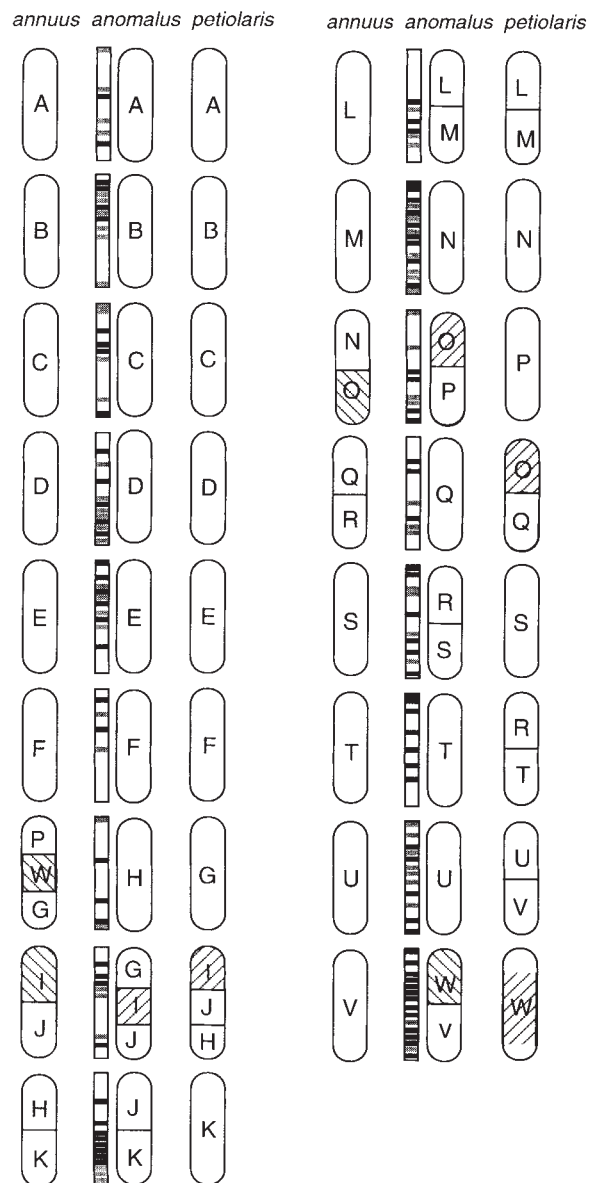


FIG. 2 Inferred structural relationships of chromosomes of the three *Helianthus* species, based on comparative linkage mapping. Lines of shading within chromosomes indicate inversions. Vertical rectangles adjacent to *H. anomalus* chromosomes indicate the distribution of markers from the two parental species: *H. annuus* and *H. petiolaris* markers are indicated by black and grey bars, respectively.

In addition to confirming theoretical genetic models for speciation by hybrid recombination, this study demonstrates the utility of comparative linkage mapping for studying the genomic processes accompanying or facilitating different modes of speciation. Not only can these maps be used to infer precisely the genomic structural changes accompanying speciation, but they also allow the genomic contribution of parental taxa to hybrid species or introgressant populations to be determined on a chromosome by chromosome basis, as well as within chromosomes. Future comparative mapping studies of species-level questions could be enhanced by the development of microsatellite loci²⁰, because most of these loci are likely to be polymorphic even in very narrow intraspecific mapping populations, and homology among loci can be assumed without additional experiments such as those required here (Fig. 1 and Supplementary Information). Microsatellite markers are currently being developed for *Helianthus* mapping populations. □

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Defective haematopoiesis in fetal liver resulting from inactivation of the EKLf gene

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ERYTHROID Krüppel-like factor (EKLf) was originally isolated from erythroid cell RNA by differential screening and shown to be erythroid-specific, although a low level of EKLf was found in mast cell lines^{1,2}. EKLf contains three zinc-fingers homologous to those found in the Krüppel family of transcription factors. Because it binds the sequence CCACACCCT, EKLf may affect erythroid development as a result of its ability to bind to the CAC box in

the promoter of the β -globin gene^{1,2}. Mutation of this element leads to reduced β -globin expression^{3–5} and it appears to mediate the effect of the globin locus control region on the promoter⁶. Here we inactivate the EKLf gene through insertion of a *lacZ* reporter gene by homologous recombination in embryonic stem (ES) cells. Heterozygous EKLf^{+/-} mice show that the reporter gene is expressed in a developmentally specific manner in all types of erythroblasts in the fetal liver and adult bone marrow. Homozygous EKLf^{-/-} mice appear normal during the embryonic stage of haematopoiesis in the yolk sac, but develop a fatal anaemia during early fetal life when haematopoiesis has switched to the fetal liver. Enucleated erythrocytes are formed but these do not contain the proper amount of haemoglobin. We conclude that the transcription factor EKLf is essential for the final steps of definitive erythropoiesis in fetal liver.

Using the known EKLf sequence¹, we isolated a cosmid from a 129 mouse DNA library and inserted a *lacZ* reporter gene⁷ in frame and a marker gene to select for neomycin resistance⁸ at the ATG start codon (Fig. 1). Transfection of the linearized plasmid into E14 embryonic stem cells⁹ and double selection (*neo*⁺, *tk*⁻)¹⁰ resulted in 60% homologous recombinants, as determined by Southern blot analysis of individual clones (Fig. 1). Six of the ES clones with a 70–90% normal karyotype were injected into mouse blastocysts¹¹, and three (D1, D8 and D32) resulted in chimaeric mice that transmitted the EKLf⁻ allele through the germ line (Fig. 1). The three founder lines were all bred to different background mice (FVB, BL6 and CBA). All of these combinations resulted in the same phenotype and therefore the different mice will not be discussed separately.

Analysis of the different developmental stages of the mice showed that the *lacZ* coding unit driven by the EKLf gene was first expressed in all types of erythroblasts (not shown) in the fetal liver (Fig. 2a) through to adult bone marrow. The gene was not expressed in the embryonic yolk sac or blood (as shown by the lack of LacZ staining, which does not exclude a low level of EKLf expression; Fig. 2b) or in any other tissue during

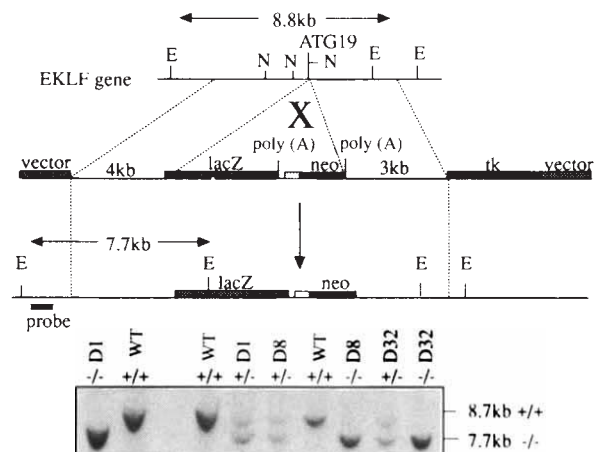


FIG. 1 Homologous recombination into the murine EKLf gene. The coding region of the bacterial *lacZ* gene⁷ and the *neo*-selectable marker¹⁰ were isolated as a 4.6-kb (*Nco*I compatible) *Bsp*HI fragment (D.M., unpublished work) and cloned into a partially digested 7-kb *Nco*I fragment at methionine 19 of the EKLf gene¹. The linearized EKLf/*lacZ/neo* plasmid, which already contained the herpes simplex virus thymidine kinase (*tk*) gene, was introduced into E14 ES cells⁹. Homologous recombinants were detected by hybridization with internal (*lacZ*) and external flanking probes. After creating chimaeric mice by ES cell injection into blastocysts¹¹, three separate founder lines were established. Bottom panel, Southern blot hybridization with a 5' flanking probe of the three founder lines. The wild-type (WT) EKLf allele is detected as an 8.7-kb fragment; the recombinant allele is detected as a 7.7-kb fragment, because of the introduction of a novel *Eco*RV site. E, *Eco*RV; N, *Nco*I.