Mapping of Hybrid Incompatibility Loci in Nasonia

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ABSTRACT

According to theory, F_2 hybrid breakdown (lethality or sterility) is due to incompatibilities between interacting genes of the different species (*i.e.*, the breaking up of coadapted gene complexes). Detection of such incompatibilities is particularly straightforward in haplodiploid species, because virgin F_1 hybrid females will produce haploid recombinant F_2 males. This feature allows for screening of the complete genome for recessive genetic incompatibilities. Crosses were performed between *Nasonia vitripennis* (v) and its sibling species *N. giraulti* (g). First, a linkage map was produced using RAPD markers. RAPD markers showed an overall bias toward *vitripennis* alleles, a pattern not predicted by the basic two-interactor Dobzhansky-Muller model. Recovery patterns of visible markers were consistent with those of linked RAPD markers. If particular genetic interactions between two loci are causing hybrid lethality, then those genotypes should be underrepresented or absent among adult F_2 males. Four sets of significant incompatibilities were detected by performing pairwise comparisons of markers on different chromosomes. Likely explanations for the observed patterns are maternal effect-zygotic gene incompatibilities or clustering of incompatibility loci. Due to the short generation time, advantages of haplodiploidy, and availability of markers, Nasonia promises to be a productive system for investigating the genetics of hybrid inviability.

HYBRIDIZATION between closely related species is a regularly observed phenomenon (*e.g.*, Bock 1984; Orr *et al.* 1994). Often, F_1 hybrid offspring are viable and fertile whereas increased mortality and sterility occur in the second (F_2) generation (*e.g.*, closely related Drosophila species; Wu and Palopoli 1994; Hutter 1997). This phenomenon is referred to as "hybrid breakdown." Both F_1 hybrid problems and F_2 hybrid breakdown are thought to be caused by differential fixation of alleles in two species which interact negatively in hybrids, *i.e.*, are incompatible (Dobzhansky 1937; Muller 1942). Several hypotheses have been proposed to explain increased mortality of F_2 relative to F_1 hybrids, but the currently most favored explanation is that negative epistatic interactions between loci of the species tend to be recessive (Muller 1942; Orr 1993).

Typically, two approaches have been used to study the underlying genetics of hybrid breakdown: (1) examining F_2 backcross hybrids between closely related species and estimating the number of genetic "factors" involved in hybrid breakdown by testing for viability or fertility effects (*e.g.*, Dobzhansky 1936; Coyne 1992); and (2) introgressing a defined chromosomal segment from one species into the genetic background of a closely related species and studying the effects of this particular segment on viability or fertility (*e.g.*, Cabot *et al.* 1994; True *et al.* 1996). Most genetic analyses on hybrid breakdown in insects are done in Drosophila species [for a review of the Drosophila literature see Powell (1997)]. Much less is known about the genetic basis of hybrid breakdown in other insect species (but see Barton and Hewitt 1985; Harrison 1990; Beeman *et al.* 1992; Breeuwer and Werren 1995).

Hybrid breakdown in the parasitoid wasp genus Nasonia has been studied intensively (Werren 1991; Breeuwer and Werren 1995). The genus includes three closely related species, which are thought to have split into separate species between 0.1 and 0.2 mya (Campbell *et al.* 1993). Two species, *Nasonia vitripennis* and *N. giraulti*, occur sympatrically in eastern North America (Darling and Werren 1990). These species are normally reproductively isolated because of a cytoplasmically inherited rickettsia of the genus Wolbachia. However, *N. vitripennis* and *N. giraulti* can produce viable hybrid offspring when they are cured of their Wolbachia parasites with antibiotics (Breeuwer and Werren 1990). F_1 hybrid females show only a slightly reduced viability but are otherwise healthy and fecund (Breeuwer and Werren 1995). Mortality increases significantly in both sexes of the second generation (Breeuwer and Werren 1995) leading to a significant hybrid breakdown. However, mortality is much greater among (haploid) F_2 males than among F_2 females produced by backcrossing to either parental species. This result is interpreted as indicating that incompatible interactions are generally recessive in this species cross.

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Breeuwer and Werren (1995) have shown that both nucleo-cytoplasm and nucleo-nucleotype interactions are involved in the hybrid breakdown of interspecific crosses between *N. vitripennis* and *N. giraulti.* The nucleo-cytoplasm interaction is asymmetric, *e.g.*, F_2 hybrid males suffer a significantly increased mortality (50%) if in a *N. giraulti* cytoplasm, compared to a *N. vitripennis* cytoplasm. Breeuwer and Werren (1995) introgressed the *N. giraulti* nuclear genes into a *N. vitripennis* cytoplasm. This introgression strain showed the same viability and fertility as a normal *N. giraulti* strain. However, the reciprocal introgression of *N. vitripennis* nuclear genes in a *N. giraulti* cytoplasm suffered from a constant high mortality (≈90%), severe enough to cause the termination of the introgression experiment after the fifth generation, indicating an extreme incompatibility between *N. giraulti* cytoplasm and *N. vitripennis* nuclear genes.

With the advent of new molecular marker techniques [*e.g.*, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites] and mapping programs, it is now possible to generate genetic linkage maps quickly and efficiently for nonmodel organisms (*e.g.*, Hunt and Page 1995; Antolin *et al.* 1996; Laurent *et al.* 1998). These developments also make it possible to try a new approach to estimate the genetic basis and number of genes involved in hybrid breakdown in Nasonia. Hymenoptera have a haplodiploid sex determination mechanism; females produce males parthenogenetically and males are, therefore, haploid. A haploid mapping population has two major advantages: (1) dominant molecular markers like those expressed as RAPD are always informative and (2) there are no dominance interactions between alleles of the same locus and, therefore, epistatic viability effects can be directly assessed, and potential sets of interacting loci causing hybrid mortality can be readily mapped.

Objectives of this study were to generate a RAPD linkage map of Nasonia and to identify and map sets of interacting loci involved in F_2 hybrid breakdown between *N. vitripennis* and *N. giraulti.* Crosses were performed between *N. vitripennis* males and *N. giraulti* females (with an *N. vitripennis* cytoplasm). The resulting F_1 hybrid females produced parthenogenetically a population of F_2 haploid recombinant males. These were used to generate a RAPD linkage map and to identify negative epistatic interactions contributing to F_2 hybrid mortality. Results confirm that this approach can be used to quickly identify candidate regions involved in hybrid breakdown, especially between haplodiploid species.

MATERIALS AND METHODS

Nasonia stocks: All Nasonia strains used were highly inbred and cured of their Wolbachia parasites by antibiotics, because only cured strains will produce viable and fertile F_1 hybrids (Breeuwer and Werren 1990). The *N. vitripennis* strain AsymC is derived from a wild-type strain (LabII). The *N. giraulti* strain R16A was created by 16 generations of backcrossing *N. giraulti* males to *N. vitripennis* females, resulting in a mostly *N. giraulti* nuclear genome in a *N. vitripennis* cytoplasm (Breeuwer and Werren 1995). To produce F_2 males for mapping, females of the R16A strain were crossed with males of AsymC. Fifteen F_1 hybrid females derived from one pair produced 178 males, which were used to construct the genome map. Males from multiple families could be combined into one mapping population because both strains are highly inbred and were homozygous for all markers.

Mutant strains were used to place visible mutants from each linkage group of Nasonia onto the RAPD molecular map. The strains employed were the "R locus" allele *red 833* (G. B. Saul, unpublished results), *rdh 5*, *bk 424*, *or 123*, and *st 318*, which occur on linkage groups I, II, III, IV, and V, respectively (Saul 1993). Males of the mutant strains were crossed with females of R16A and F_1 females were placed on hosts as virgins to produce F_2 males for mapping. Haploid males derived from hybrid females segregated for wild-type and mutant phenotypes. Six wild-type and six mutant males derived from each cross were screened with 20 RAPD primers to find mapped markers segregating with the phenotypes. For *bk 424* an increased sample size of mutant and wild-type males ($n = 18$) were tested for markers that segregated with the phenotype in the first screen.

DNA extraction and PCR reactions: DNA from thorax and gaster of individual males and females was isolated with a standard CTAB-Phenol extraction method (Hunt and Page 1995). Head and wings from males of the mapping population were retained for further quantitative trait locus (QTL) studies. RAPD-PCR reactions (Williams *et al.* 1990) were carried out in 12.5-μl reaction volumes using 5 ng of genomic DNA, 0.6 μm primer, 100 μm each dATP, dCTP, dGTP, and dTTP (Pharmacia, Piscataway, NJ), 10 mm Tris-HCL (pH 8.3), 50 mm KCl, 2 mm $MgCl_2$, and 0.25 units Taq.

The 10 nucleotide primers were obtained from Operon Technologies (Alameda, CA) or the University of British Columbia Biotechnology Center (Vancouver, Canada). Amplification was performed with the following cycle parameters: 5 cycles of 94°/1 min, 35°/1 min, and 72°/2 min; another 32 cycles at 94°/10 sec, 35°/30 sec, and 72°/30 sec.

Gel electrophoresis and scoring: The amplification products were resolved in 20 × 25-cm horizontal gels using 1% Synergel (Diversified Biotech, Newton Center, MA) and 0.6% Agarose in a 0.5× TBE buffer (Sambrook *et al.* 1989). Gels were run for 500–600 V hr, stained in ethidium bromide for 25 min, destained in distilled water for another 40 min, and recorded on an UV transilluminator with Polaroid 667 films. The gels were read and the results directly entered into an Excel spreadsheet. After the map and the linkage groups were established all markers were ordered according to their position in the linkage groups and all gels were scored a second time. This provided a control for unlikely double crossovers due to scoring errors.

Linkage analyses: MAPMAKER (Lander *et al.* 1987; version 2.0 for the Macintosh) was used to analyze the data and generate a linkage map. The data type was coded as "haploid." The mapping procedure followed a standard protocol described below.

1. A two-point linkage analysis of the whole data set (105 marker) was performed with the "GROUP" command (setting: LOD = 5, θ = 0.25) to find a preliminary set of linkage groups.
2. Multi-point analysis within all putative linkage groups was done with the "FIRSTORDER" command (LOD = 5, θ = 0.25). This analysis gave the most likely order of the markers in each linkage group.

3. Using the "RIPPLE" command, the order found in step 2 was tested within each linkage group for all possible three-point orders of consecutive markers. The most likely order for every marker is shown. Markers that were linked at 2 cM or less could not be ordered at a LOD 2 threshold because with the given size of the mapping population there are too few informative meioses.

All map distances (cM) were calculated from recombination fractions (%) according to Kosambi's mapping function (Kosambi 1944). Kosambi's function resulted in less map expansion than Haldane's when the "DROP MARKER" command was performed.

Analysis of mtDNA: A part of the mitochondrial COI gene (385 bp) of all three Nasonia species (*N. giraulti*, *N. vitripennis*, and *N. longicornis*) was amplified with universal primers and sequenced to confirm that the *N. giraulti* R16A strain contained *N. vitripennis* cytoplasm. DNA was isolated as described above and the following primers were used to amplify the mtDNA: 5′ CAA CAT TTA TTT TGA TTT TTT 3′ and 5′ GCW ACW ACR TAA TAK GTA TCA TG 3′ (developed by Ted Schulz). PCR reactions were carried out in 25-μl reaction volumes using 5 ng of genomic DNA, 2 μm primer, 100 μm each dATP, dCTP, dGTP, and dTTP (Pharmacia), 10 mm Tris-HCL (pH 8.3), 50 mm KCl, 2 mm $MgCl_2$, and 0.25 units Taq. Five microliters of the PCR reaction was used to control whether the amplification was successful and the remaining 20 μl was purified with Microcon 100 microconcentrators (Amicon, Beverly, MA). Sequencing was done in the DBS automated sequencing facility of the University of California, Davis. For the sequencing reaction the ABI PRISM Big Dye terminator cycle sequencing reaction kit (Perkin-Elmer/ABI, Weiterstadt, Germany) was used. The reactions were run on an ABI Prism 377 DNA sequencer using a 5% Acryl/Bisacryl Long Ranger gel. Data were analyzed using ABI Prism sequencing 2.1.1. software.

Sequences were obtained from two males of *N. giraulti* (strain: 233F), one male of *N. giraulti* with *N. vitripennis* cytoplasm (strain: R16A), *N. vitripennis* with *N. vitripennis* cytoplasm (strain: AsymC), and *N. longicornis* (strain: RNLCA9304), respectively. Sequences were proofread by comparing the results of both primers, aligned by eye, and then analyzed with PAUP (Version 3.1.1. for Apple Macintosh, written by David L. Swofford).

Comparing maps: The map size of the interspecific cross was compared with the previously published intraspecific map of *N. vitripennis* (Saul and Kayhart 1956; Saul 1993). For this purpose the recombination fractions (%) given by Saul and Kayhart (1956) and Saul (1993) had to be transformed to map units (cM) using Kosambi's map function [map unit (cM) $= -\ln((1 + 2y)/(1 - 2y))$, where y is the recombination fraction; Kosambi 1944]. Kosambi's map function was used because the interspecific map was constructed using Kosambi's map function.

There is a problem in comparing map sizes. Most of the published maps for nonmodel organisms are not saturated and, therefore, we do not have estimates of actual map sizes. One possibility is to compare map size as a function of number of markers for the different species. One way to compare is to construct maps with equal numbers of markers and compare their sizes. For instance, a larger genome would yield a larger map size with the same number of markers. We used increasing numbers of randomly selected markers from two different maps, *Apis mellifera* and Nasonia, and constructed maps using MapMaker (Lander *et al.* 1987). We then calculated the total map size, which was the size of each linkage group plus an added 40 cM for all unlinked markers. Our justification for adding 40 cM is that we knew all markers were linked somewhere, but were left unlinked because they had no companion markers within 40 cM of them ($\theta = 0.40$). Our expectation is that genomes with more recombination will be relatively much larger with a small number of markers because fewer markers are likely to be linked together into linkage groups. As marker numbers increase, linkage groups will coalesce, resulting in a slower increase in map size as the map approaches saturation.

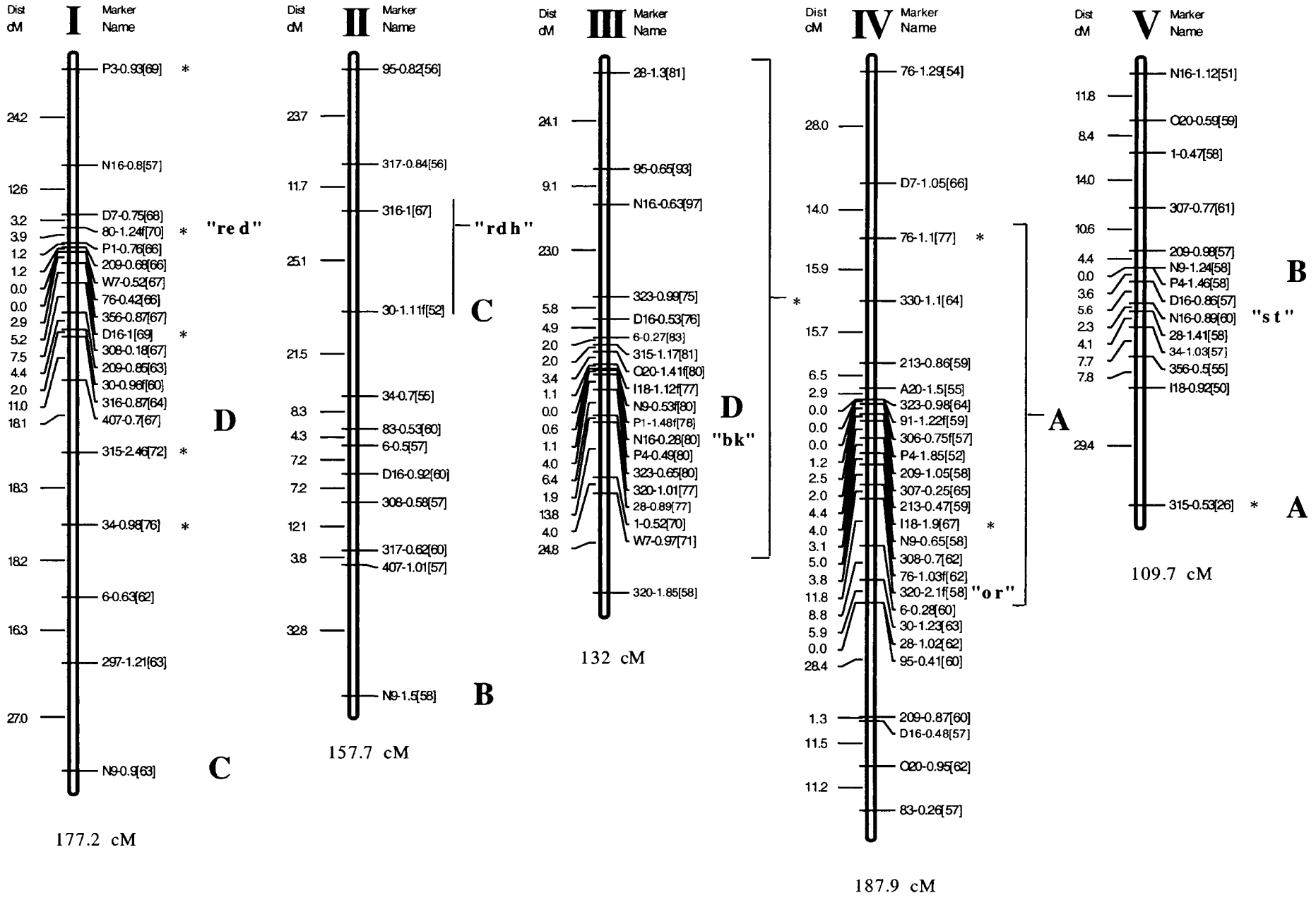
To compare the map size of *A. mellifera* (Hunt and Page 1995) and Nasonia, the linked markers of both data sets (*A. mellifera*, 365 markers and Nasonia, 91 markers) were randomized and linkage of 20, 40, 60, 80, and 91 markers were then calculated. For the mapping procedure we used MAPMAKER (Lander *et al.* 1987; version 2.0 for the Macintosh) with the following default settings: $\theta = 0.40$; LOD = 3.0; Kosambi mapping function.

Statistical analysis: Segregation distortion was tested with a chi-square test for goodness of fit to the expected segregation ratio of 1:1. Epistatic interactions were detected with chi-square tests performed between the observed and expected distribution of recombinant and nonrecombinant genotypes between every marker from different linkage groups ($n = 3252$). If selective mortality of F_2 males is due to epistatic interaction between genes or gene complexes of the two species, a significant decrease in the observed recombinant genotypes should be detected. A Bonferroni correction was used to correct for the overall type I error by dividing $\alpha = 0.01$ by the total number of comparisons done (3252). Therefore, our rejection criteria for the null hypothesis was $P = 0.000003$ for each comparison. This is an extremely conservative approach, because comparisons are not all independent due to linkage of markers on the five chromosomes.

RESULTS

Genome map: A total of 522 RAPD primers were tested on both parental strains (R16A and AsymC) and the hybrid F_1 females. The best 39 primers were used for mapping, *i.e.*, primers that were polymorphic between the two parental strains and showed Mendelian inheritance. The screening allowed us to assign phase to every marker, *i.e.*, we knew for every marker whether it came from *N. vitripennis* or *N. giraulti*. The 39 primers generated 105 variable marker bands (2.7 markers per primer) segregating in the mapping population of 178 males. Most of the markers showed presence-absence polymorphism (89.5%) whereas 10.5% (11 markers) were fragment-length polymorphisms. Ninety-one markers (87%) could be mapped into five linkage groups that spanned over 764.5 cM (Figure 1). *N. vitripennis* and *N. giraulti* both have five chromosomes and the five linkage groups most likely represent the five chromosomes of Nasonia. The average distance between 2 markers in the linkage map was 8.4 cM.

Markers show significant clustering on four of the five linkage groups (two-tailed Kolmogorov-Smirnov test against a uniform distribution: linkage group (LG) I—$P = 0.029$, $Z = 1.455$; LG II—$P = 0.135$, $Z = 1.161$; LG III—$P = 0.001$, $Z = 1.969$; LG IV—$P < 0.001$, $Z = 2.056$; LG V—$P = 0.011$, $Z = 1.611$). We suspect that such clusters represent the centromeric regions of these chromosomes. Both centromeres and regions flanking cen-


Dist
cM
I
Marker
Name
P3-0.93[69] *
24.2
N16-0.8[57]
12.6
D7-0.75[68]
3.2
80-1.24f[70] *
3.9
P1-0.76[66]
1.2
209-0.68[66]
1.2
W7-0.52[67]
0.0
76-0.42[66]
0.0
356-0.87[67]
2.9
D16-1[69] *
5.2
308-0.18[67]
7.5
209-0.85[63]
4.4
30-0.96[60]
2.0
316-0.87[64]
11.0
407-0.7[67]
18.1
315-2.46[72] *
18.3
34-0.98[76] *
18.2
6-0.63[62]
16.3
297-1.21[63]
27.0
N9-0.9[63]
"red"
D
C
177.2 cM
Dist
cM
II
Marker
Name
95-0.82[56]
23.7
317-0.84[56]
11.7
316-1[67]
25.1
30-1.11f[52]
21.5
34-0.7[55]
8.3
83-0.53[60]
4.3
6-0.5[57]
7.2
D16-0.92[60]
7.2
308-0.58[57]
12.1
317-0.62[60]
3.8
407-1.01[57]
32.8
N9-1.5[58]
"rdh"
C
B
157.7 cM
Dist
cM
III
Marker
Name
28-1.3[81]
24.1
95-0.65[93]
9.1
N16-0.63[97]
23.0
323-0.99[75]
5.8
D16-0.53[76]
4.9
6-0.27[83]
2.0
315-1.17[81]
2.0
C20-1.41f[80]
3.4
I18-1.12f[77]
1.1
N9-0.53f[80]
0.0
P1-1.48f[78]
0.6
N16-0.28[80]
1.1
P4-0.49[80]
4.0
323-0.65[80]
6.4
320-1.01[77]
1.9
28-0.89[77]
13.8
1-0.52[70]
4.0
W7-0.97[71]
24.8
320-1.85[58]
*
D
"bk"
132 cM
Dist
cM
IV
Marker
Name
76-1.29[54]
28.0
D7-1.05[66]
14.0
76-1.1[77] *
15.9
330-1.1[64]
15.7
213-0.86[59]
6.5
A20-1.5[55]
2.9
323-0.98[64]
0.0
91-1.22f[59]
0.0
306-0.75f[57]
0.0
P4-1.85[52]
1.2
209-1.05[58]
2.5
307-0.25[65]
2.0
213-0.47[59]
4.4
I18-1.9[67] *
4.0
N9-0.65[58]
3.1
308-0.7[62]
5.0
76-1.03f[62]
3.8
320-2.1f[58]
11.8
6-0.28[60]
8.8
30-1.23[63]
5.9
28-1.02[62]
0.0
95-0.41[60]
28.4
209-0.87[60]
1.3
D16-0.48[57]
11.5
C20-0.95[62]
11.2
83-0.26[57]
"or"
A
187.9 cM
Dist
cM
V
Marker
Name
N16-1.12[51]
11.8
C20-0.59[59]
8.4
1-0.47[58]
14.0
307-0.77[61]
10.6
209-0.98[57]
4.4
N9-1.24[58]
0.0
P4-1.46[58]
3.6
D16-0.86[57]
5.6
N16-0.89[60]
2.3
28-1.41[58]
4.1
34-1.03[57]
7.7
356-0.5[55]
7.8
I18-0.92[50]
29.4
315-0.53[26] *
B
"st"
A
109.7 cM

tromeres are typically composed of large stretches of tandem repetitive DNA and also are known to accumulate mobile elements at rates higher than euchromatin (Charlesworth *et al.* 1994). Inversions within tandem arrays will create matching oligomeric sequences in opposite orientation, thus resulting in the conditions necessary for RAPD amplification. However, a more likely explanation is that recombination rates in regions flanking centromeres are reduced, which results in longer stretches of DNA per recombination unit. This effect will also increase the number of molecular markers expected per map unit in centromeric regions. Independent results also support the occurrence of centromeres in the regions showing clustering of RAPD markers. The *red 833* allele maps to the R locus of Nasonia, which is known to occur near the centromere based upon suppressed recombination rates and production of centric fragments containing this locus (Ryan *et al.* 1985). Mapping studies (below) place the *red 833* locus near the cluster of RAPD markers on this linkage group. Similarly, centric fragments have been generated that contain the *or 123* locus (F. Perfetti, J. H. Werren and M. J. Perrot-Minnot, unpublished results) and the *or 123* locus maps close to the cluster of RAPD markers found on chromosome IV.

Placing visible markers on the RAPD map: Approximately 20 strains with visible mutant markers are currently available in *N. vitripennis*, most of which are eye color mutants (Saul 1993). Visible mutations from each of the five linkage groups of *N. vitripennis* (Saul 1993) were selected to be mapped onto the RAPD linkage map, *red 833* (linkage group I), *rdh 5* (linkage group II), *bk 424* (linkage group III), *or 123* (linkage group IV), and *st 318* (linkage group V). Four phenotypic markers, *red 833* (Fisher's exact $P = 0.015$, 6 wild-type and 6 mutant males), *bk 424* (Fisher's exact $P = 0.025$, $n = 8$ wild-type and 10 mutant males), *or 123* (Fisher's exact $P = 0.002$, 6 wild-type and 6 mutant males), and *st 318* (Fisher's exact $P = 0.002$, 6 wild-type and 6 mutant males), showed significant linkage with markers on four different linkage groups (Figure 1). The mutation *rdh 5* showed no significant segregation with a specific marker but was determined to be on linkage group II by a process of elimination. Within linkage group II, *rdh 5* showed closest linkage to 2 markers (316-1 and 30-1.11f), which are located in a region of low marker density (Figure 1). As expected, the mutant *red 833* mapped to a region on linkage group I that showed a low recombination rate (based upon the observed high density of RAPD markers; Figure 1). This is expected because *red 833* is present in the R locus (Whiting 1967), a region of low recombination that is known to be near the centromere (Saul 1993; J. H. Werren, unpublished results). Mutant *or 123* segregated in the initial screen with a region on linkage group IV spanning almost 55 cM 6 markers using 12 individuals). Finer-scale mapping indicates that *or 123* is more closely linked to the marker 320-2.1f (Figure 1). This was accomplished by backcrossing the wild-type *giraulti* allele into *vitripennis* for 10 generations, each generation selecting the heterozygous ($+_g/or_v$) females for crossing to or_v males. Screening 13 markers in the 55-cM region, only marker 320-2.1f retained a *N. giraulti* allele in phenotypically wild-type males. All other markers were fixed for *N. vitripennis* alleles in males of both phenotypes. This demonstrated the tight linkage of *or 123* and this marker.

Unequal recovery of markers in hybrid males: Due to meiosis in hybrid females equal frequencies of the *N. giraulti* (g) and *N. vitripennis* (v) alleles are expected for each marker among their sons, assuming that markers are not associated with hybrid lethality or meiotic drive. However, the recovery ratios of 90 of the 91 mapped markers among adult F_2 males were biased toward *N. vitripennis* alleles and 47 markers on the map were significantly so ($P < 0.05$; chi-square tests results not shown; Figure 1). After a Bonferroni correction ($\alpha' = 0.05 = \alpha/91$) 26 markers were still significantly distorted (* in Figure 1). Markers with significant recovery distortion were mainly distributed on two linkage groups (I + III; Figure 1). Eighteen of 19 markers on linkage group III were significantly distorted, which made up for >64% of the significantly distorted markers (18/26) in the interspecific Nasonia map. Only 1 mapped marker and 3 of the unmapped markers showed a bias toward g alleles (Figure 1). Overall, 96% of the genome was biased toward *N. vitripennis* markers.

It is possible that the observed marker bias was due to particular individuals who had extraordinarily high representations of *N. vitripennis* marker alleles. To test that, we plotted the recovery rate of *N. vitripennis* alleles for each male. The recovery rate showed a normal distribution with mean 0.65 and lower and upper 95% confidence intervals of 0.640 and 0.691, respectively (Figure

Figure 1.—Linkage map of an interspecific cross between *N. vitripennis* and *N. giraulti* based on RAPD markers. Marker names are composed as follows: (1) The RAPD primer name is a letter and a number for Operon primer (Operon Technologies) or a number only for primers purchased from the University of British Columbia (Vancouver); (2) after the dash is the approximate size of the marker band in kilobases; and (3) in brackets is the bias, in percentage, toward the *N. vitripennis* allele. If the marker is a fragment-length polymorphism, the size of the marker is followed by an f. All markers mapped with a minimum LOD score of 5.0 and a θ of 0.25. The mapped phenotypic markers (*red 833, bk 424, or 123,* and *st 318*) are shown next to the markers with which they showed a significant linkage. The phenotypic marker *rdh 5* is shown next to the marker with which it showed the closest association (see results). The markers or genome regions displaying significant negative epistatic interactions are designated with capital letters, *i.e.*, A interacts with A and so on (see Table 2). Markers that showed a significant unequal recovery rate ($\alpha' = 0.05$) after Bonferroni correction are marked with an asterisk (*).

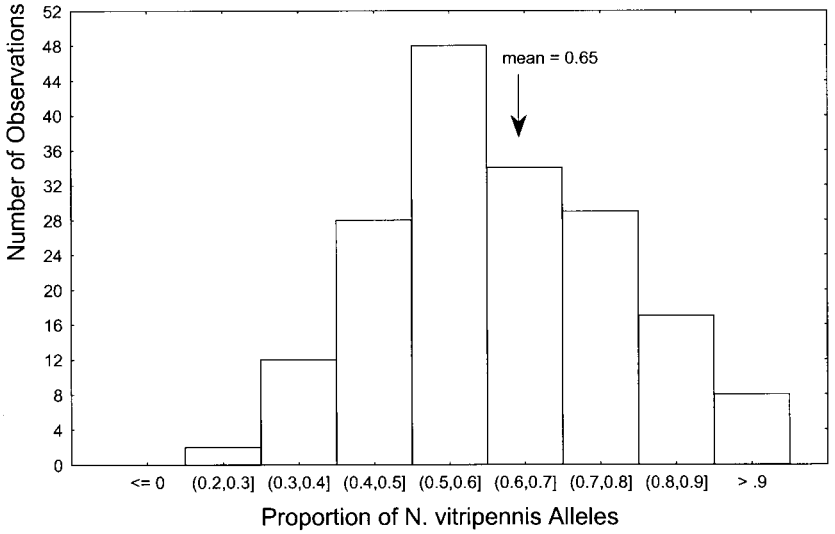


Figure 2.—Percentage of *N. vitripennis* alleles in F_2 hybrid males. The percentage of *vitripennis* alleles in each of the 178 males of the mapping population are shown. The mean was 64.9% (95% confidence intervals 0.64–0.69).

2; confidence intervals are not shown). Confidence intervals were calculated from arcsine square root transformed data by multiplying the standard error of the transformed data by $t_{0.05[177]}$ and then adding and subtracting from the mean. Those values were then back transformed yielding confidence limits (Sokal and Rohlf 1995, p. 147, box 7.2; see also Figure 2). The confidence limits do not span 0.50, demonstrating that the recovery rate is biased from 1:1 with an excess of markers from *N. vitripennis*. In general males surviving the hybrid breakdown have a significantly higher proportion of *N. vitripennis* alleles. Therefore, the bias toward *N. vitripennis* alleles is a general phenomenon and not due to a particular group of males. Test crosses with three *N. vitripennis* strains carrying visible markers (*st 318*, *or 123*, and *bk 424*) showed unequal recovery rates similar to the RAPD markers to which they were linked (Figure 1; Table 1).

Possible causes of this overall bias are discussed in more detail later. However, one possibility is an incompatibility between *N. giraulti* nuclear genes and *N. vitripennis* cytoplasmic genes (*e.g.*, mitochondria). The assumption that *N. giraulti* nuclear genes show no negative interaction with the *N. vitripennis* cytoplasm is inferred from the successful introgression of *N. giraulti* nuclear genome into a *N. vitripennis* cytoplasm (Breeuwer and Werren 1995). However, it could be that the introgressed strain was contaminated with *N. giraulti* cytoplasm by some laboratory error, *i.e.*, what was assumed to be a *N. giraulti* genome in a *N. vitripennis* cytoplasm was really a *N. giraulti* genome in a *N. giraulti* cytoplasm. To exclude this possibility, a part of the mitochondrial COI gene (385 bp) was sequenced to confirm that the introgression strain has a *N. vitripennis* cytoplasm or at least a *N. vitripennis* mitotype. Sequences of two males from each of the *N. vitripennis* (strain AsymC), *N. giraulti* (strain 233F), *N. longicornis* (strain RNLCA9304), and the introgression strain R16A were obtained. When we compared the COI sequences of *N. vitripennis* and *N. giraulti* we found a total 34 nucleotide exchanges (8.83%). The sequences from the two sequenced individuals of *N. giraulti* (strain 233F) were identical as well as the sequences from AsymC (*N. vitripennis*) and R16A (introgression strain). Hence, the introgressed strain R16A has a cytoplasm containing the *N. vitripennis* mitochondria, as expected.

TABLE 1

Comparison of recovery rates of phenotypic (mutant) and linked RAPD markers among F_2 hybrid males

Cross (male × female)	F_2m	F_2+	% m	RAPD marker	% vit
st318 × R16A	799	652	55	N16-0.89	60
or123 × R16A	941	664	59	320-2.1f	58
bk424 × R16A	459	153	75	N16-0.28	80

Males of three different *N. vitripennis* strains carrying phenotypic markers (*st 318*, *or 123*, and *bk 424*) were crossed with the *N. giraulti* strain R16A. The frequency of phenotypes (F_2+, wild type; F_2m, mutant) of F_2 haploid males and the corresponding frequency of tightly linked RAPD markers are shown. The RAPD and mutant marker frequencies were determined from separate crossing populations and therefore are independent samples.

Epistatic interactions: Epistatic interactions between nuclear genes are thought to be the main cause of the observed hybrid breakdown in males from a hybrid cross of *N. vitripennis* and *N. girault*i in a *N. vitripennis* cytoplasm (Breeuwer and Werren 1995). Hybrid breakdown in Nasonia results in 50–80% of F_2 male offspring dying during the larval and pupal stages (Breeuwer and Werren 1995). If the observed mortality is due to negative epistatic interactions between nuclear genes of the two species, specific recombinant genotypes should be missing among the surviving adult males. To test for this effect, we conducted chi—square tests for combinations of markers across the genome. To avoid the complications of linkage, only markers from different linkage groups were examined for negative epistatic interactions. A total of 3252 pairwise chi-square tests between all markers of different linkage groups of our map were performed and the analyses were conservatively corrected by the Bonferroni method under the assumption of complete independence of comparisons (see materials and methods). Using this approach, we detected many interacting regions. However, only four marker pairs showed a significant deficiency of recombinant genotypes after a Bonferroni correction (*i.e.*, $\alpha' = 0.01 = \alpha/3525$). Three show a deficiency of the recombinant (vg or gv) genotypes and one pair shows a deficiency of the nonrecombinant genotype (gg).

The marker pairs showing significant reduction of recombinant genotypes are shown in Figure 1 and Table 1. Pair A involves an interaction between regions on linkage groups IV and V. In this pair, there is a complete absence of the gv marker pair, whereas the reciprocal recombinant vg occurs at frequencies comparable to the parental combinations (gg and vv). This is exactly the pattern predicted by the Dobzhansky-Muller model for the early stages of speciation (Dobzhansky 1937; Muller 1942). Pair B involves interactions between markers on linkage groups V and II and shows a significant deficiency in both recombinant types (vg and gv). This pattern is not predicted by the basic two-locus Dobzhansky-Muller model. Pair C involves interactions between a marker pair on linkage groups II and I. This combination shows significant deficiencies in both recombinant categories, with a greater reduction in the vg combination. Finally, pair D shows a strong and unexpected effect; interactions between genes linked to markers on linkage groups I (407-.07) and III (N9-0.53f) result in a complete absence of the parental genotype gg. This result is totally unexpected and is not predicted by the standard two-interactor genetic incompatibility model. Possible explanations for these patterns are explored in the discussion.

Map size and recombination frequency: Size of the interspecific Nasonia map can be compared to map sizes of other species. For this purpose, two other parasitic and one eusocial hymenopteran species maps are used that had also been constructed with RAPD markers [*Bracon hebetor*, Antolin *et al.* (1996); *Trichogramma brassicae*, Laurent *et al.* (1998); *A. mellifera*, Hunt and Page (1995); Figure 3]. Additionally, two smaller intraspecific maps of *N. vitripennis* (Saul and Kayhart 1956; Saul 1993) are included (Figure 3). However, these two maps were based on visible markers.

The intra- and interspecific maps of Nasonia are the smallest linkage maps in Hymenoptera so far reported (Figure 3). The interspecific Nasonia map has many fewer markers than the *A. mellifera* map (91 compared to 365), nevertheless, the average interval size between markers is similar between them (Nasonia map, 8.4 cM and the *A. mellifera* map, 9.1 cM). This is especially interesting because the physical size of the *N. vitripennis* genome is nearly twice as large as that of *A. mellifera* [*N. vitripennis* is 312 Mb, Rasch *et al.* (1977); *A. mellifera* is 178 Mb, Jordan and Brosemer (1974)]. Because map distances are based on recombination frequency, this also suggests a much smaller recombination frequency in the interspecific Nasonia map, compared to the *A. mellifera* map. Therefore, the relationship between physical distance and map unit (cM) is eight times higher in the interspecific Nasonia map (0.41 Mb/cM) compared with the Apis map [0.05 Mb/cM, Hunt and Page (1995)]. These values (0.41 and 0.05, respectively) are a good relative representation of the relationship between physical and recombinational genome size of both species because the average distance between markers is equal in both maps.

Estimated map sizes were compared by regressing them against the number of markers used. The regression lines for each and their 95% confidence intervals demonstrate that the estimated map sizes of the interspecific Nasonia map were smaller for any given number of markers than those of *A. mellifera* (Figure 3). The 95% confidence intervals of both regression lines overlapped only in the region of the first 20 markers. The intraspecific *N. vitripennis* map sizes are both smaller than the interspecific Nasonia map, suggesting that recombination is not reduced in the interspecific cross. The difference in recombination frequency between Nasonia and Apis may be explained, in part, by the difference in chromosome number (5 for Nasonia and 20 for Apis). Even if chiasmata frequency per chromosome is the same for both species, a fourfold increase in recombination rate is expected in Apis.

The map sizes of the other two parasitic Hymenoptera, *T. brassicae* and *B. hebetor*, are also significantly smaller than the *A. mellifera* map, suggesting a smaller recombination frequency in all three parasitic Hymenoptera from three different hymenopteran families (Figure 3). Note that the *T. brassicae* map units were calculated by using the Haldane mapping function whereas all other map sizes were calculated using the Kosambi mapping function. Altogether these results show that the recombination frequencies in parasitic

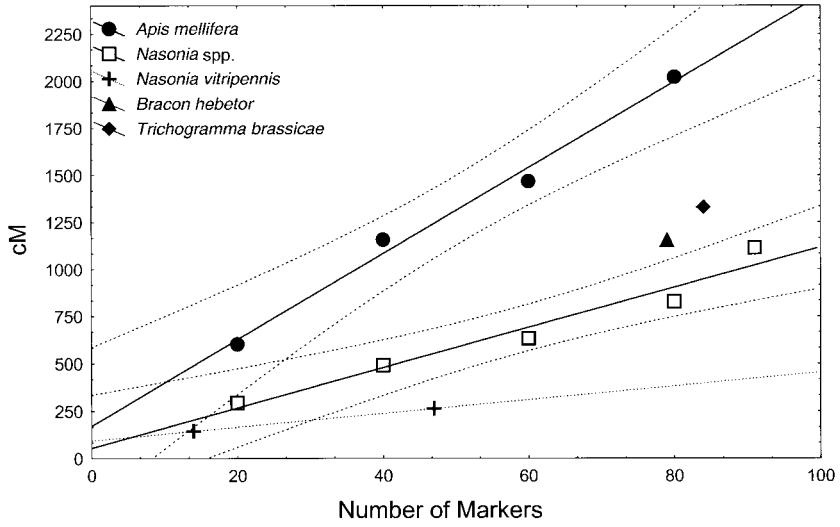


Figure 3.—Comparison between relative map sizes of different hymenopteran species. The relative map sizes for *A. mellifera* and the interspecific Nasonia linkage map were calculated for 20, 40, 60, 80, and 91 markers (for details of the calculation see materials and methods). The data for map sizes of *N. vitripennis* (Saul 1993; Saul and Kayhart 1956), *B. hebetor* (Antolin *et al.* 1996), and *T. brassicae* (Laurent *et al.* 1998) were taken from the literature.

Hymenoptera are reduced compared to *Apis mellifera.* They also demonstrate that high rates of recombination are not generally associated with a haplodiploid life cycle as proposed by Hunt and Page (1995).

DISCUSSION

A number of interesting results come from this study. First, we show that interspecies molecular marker maps can quickly be produced in haplodiploids by taking advantage of male haploidy. Second and more important, mapping techniques can be used to screen the entire genome for epistatic interactions causing lethality in F_2 hybrid males. To our knowledge, this is the first case in any organism of a total genomic screen for sets of interacting loci involved in F_2 hybrid lethality. Despite the numerous studies of hybrid inviability and sterility in Drosophila species, most attention in such studies has focused on loci located on the X chromosome (the effectively haplodiploid component of the genome), and little is known about the location of interacting loci in the autosomes (Coyne 1992). True *et al.* (1996) and Hollocher and Wu (1996) screened for autosomal hybrid lethals in the *Drosophila simulans* clade, by introgression of regions from one species into the genetic background of the other. However, these studies did not determine the specific regions interacting with these autosomal lethal factors. Features of haplodiploidy greatly facilitate quick identification of regions involved in specific interactions resulting in genetic incompatibilities. We have found several patterns not consistent with the simple two-locus model for hybrid inviability, including an overall bias in recovery of markers among F_2 males toward one species and particular interactions inconsistent with the basic two-locus model. Finally, we have found that recombination map size of Nasonia is considerably smaller than that observed in other hymenopterans, particularly that of the honeybee (*A. mellifera*), despite the fact that Nasonia has a rather large genome size (312 Mb; Rasch *et al.* 1977).

Unequal recovery of markers in hybrid males: All but one of the linked markers of the Nasonia linkage map showed a bias toward the *N. vitripennis* markers and the distribution of 47 markers was significantly different from the expected 1:1 distribution (chi-square test, $P < 0.05$). This pattern of biased recovery contrasts to nearly equal recovery ratios observed in intraspecific studies of other Hymenopteran species, such as in *A. mellifera* (Hunt and Page 1995), *B. hebetor* (Antolin *et al.* 1996), and *T. brassicae* (Laurent *et al.* 1998). We can exclude PCR artifacts as cause for the distortion in marker recovery because of the consistency of the bias toward *N. vitripennis* markers and an independent confirmation of the unequal recovery rate using phenotypic markers (Table 1).

The basic Dobzhansky-Muller model for paired epistatic interactions does not predict such a pattern. According to the model, an epistatically interacting locus from one species (*e.g.*, *N. vitripennis*) interacts negatively (*i.e.*, is incompatible) with a locus from the other species (*e.g.*, *N. giraulti*) to cause lethality. As a result, reduction in the frequency of a *vitripennis* allele at one locus should result in a complementary reduction of a *giraulti* at another locus. Some other mechanisms must be at work to cause an overall bias of F_2 recovery toward one species. There are (at least) the following possibilities: (1) meiotic drive, (2) nuclear-cytoplasmic incompatibility, (3) maternal-zygotic incompatibilities, and (4) complex (multiple) incompatibilities. Each of these is briefly considered below.

1. *Meiotic drive:* We have assumed that the distorted recovery of markers among adult F_2 males is due to the observed larval and pupal lethality. Alternatively, meiotic drive in the gametes of heterozygous F_1 females could also result in unequal recovery. There are two points arguing against this scenario. First,

maternal meiotic drive has not yet been reported in insects; all known cases of naturally occurring meiotic drive occur in male gametogenesis. Second, when F_1 females carrying a visible mutant marker (*e.g.*, or 123, st 318, or bk 424) are backcrossed to parental males, the recovery distortion disappears among the F_2 hybrid females (data not shown). This indicates that the marker distortion is due to mortality following meiosis, rather than to meiotic drive. Thus, meiotic drive is unlikely to be a general explanation for the recovery bias, although comparisons of RAPD markers between embryos and adults are needed to determine this for any specific marker.

2. *Nuclear-cytoplasmic incompatibility:* A direct interaction between a cytoplasmic factor and nuclear genes can be excluded because the *N. girault*i nuclear genome used for this cross was already in a *N. vitripennis* cytoplasm (introgression strain R16A; Breeuwer and Werren 1995). This introgression strain shows normal viability and fertility compared to a regular *N. girault*i strain (Breeuwer and Werren 1995), which should not be the case if the effects we see are due to specific interactions of a *N. vitripennis* cytoplasmic factor and *N. girault*i nuclear genes. It is possible, however, that although *N. girault*i nuclear genes can perfectly interact with a *N. vitripennis* cytoplasm if in a pure *N. girault*i nuclear genome background, they suffer from negative nucleotype-cytoplasm interaction when in the "stressed" cellular environment of a mixed nuclear genome with *N. vitripennis.* However, this cannot be due to interactions with specific *vitripennis* nuclear genes, or those would also be underrepresented in the F_2 and therefore could not explain the overall biased recovery of *vitripennis* markers.
3. *Maternal-zygotic lethal interactions:* A more likely explanation for the overall bias toward *vitripennis* alleles is a lethal interaction between maternal effect and zygotic genes. Under this scenario, a particular maternal effect locus interacts dominantly with one or more zygotic loci to cause lethality. If the dominant maternal allele is v and the zygotic locus is g, then a strong bias toward v markers in the zygotic locus and linked regions would occur. If linked v regions have negative interactions with g alleles on other chromosomes, then the effect could "cascade" to these other regions, resulting in an overall bias toward one species among the F_2. The hypothesis is testable by determining whether specific maternal-zygotic genotypes interact to cause lethality among F_3 recombinant males. Maternal effect-zygotic hybrid lethal interactions have been described or inferred in several drosophilid hybridizations (Sawamura *et al.* 1993a,b; Hutter 1997) and are believed to account for most exceptions to Haldane's rule (Wu and Davis 1993; Sawamura 1996).
4. *Complex (multiple) incompatibilities:* Although the idea formulated by Dobzhansky (1937) and Muller (1942) does not require it, the basic genetic incompatibility model considers two-locus incompatibilities. However, more complex incompatibilities (*e.g.*, involving 3, 4, or more loci) are likely to be common (Dobzhansky 1937; Muller 1942; Palopoli and Wu 1994; Carvajal *et al.* 1996), and some theoretical treatments actually predict that more complex incompatibilities can evolve more readily (Cabot *et al.* 1994; Orr 1995). According to these models a three-way interaction would result in 27 genotypes among F_2 progeny in diploids (8 among males in haplodiploids) and in the early stages of speciation only one of these combinations is likely to be lethal. Taking the simpler haploid case, for any particular interacting set, either ggv or vvg (g, *girault*i; v, *vitripennis* allele) is likely to be a lethal interaction. In the former case, two g alleles "die" for every one v allele. Extrapolating to the entire genome, if a number of multiple incompatibilities occur within the genome and stochastically the g alleles happen to be more common among the lethals, then an overall bias of the F_2 surviving genome will be toward v.

Each of these possibilities remains to be tested empirically. However, results clearly suggest that the Nasonia genome is highly interactive and likely to contain a number of lethal interactions.

Epistatic interactions between nuclear genes: Negative epistatic interactions between pairs of nuclear genes leading to mortality can be detected by the absence of the genotypes producing the lethal phenotype. Because the individuals used for the mapping procedure were haploid, we had only four possible genotypes in the tested two-way interactions, the recombinant (vg or gv) and nonrecombinant (vv and gg) genotypes. Four significant epistatic interactions between nuclear genes were detected in a genome-wide screen for missing genotypes (Table 2). This number of interacting nuclear genes is probably a minimum number as the applied Bonferroni correction is very conservative and we found many more significant interactions that did not surpass the Bonferroni rejection threshold.

Three of the interactions (A–C; Table 2) showed the expected pattern, a significant reduction of the recombinant genotypes. Those individuals that were gv or vg at markers linked to two (presumably) interacting loci were significantly underrepresented relative to the other genotypes (Table 2). One of these three (B) did not match the classic asymmetrical pattern predicted by the Dobzhansky-Muller model, in that both recombinant classes were underrepresented. The model predicts an asymmetric incompatibility because the replacing allele in each species must increase in frequency in the presence of its interactor at the other locus. If the invading allele is incompatible with the resident interactor, then it cannot invade. The occurrence of symmetric reductions in recombinants therefore suggests suc-

TABLE 2

Observed and expected distribution of genotypes of the four pairs of RAPD markers showing significant negative epistatic interactions

Observed			Marker pair	Expected		
	gir	vit	91-1.22f/		gir	vit
gir	58	0	315-0.53	gir	43.1	14.9
vit	49	37	A	vit	63.9	22.1
	gir	vit	P4-1.46 /		gir	vit
gir	27	7	N9-1.5	gir	11.7	17.3
vit	7	36	B	vit	17.3	25.7
	gir	vit	30-1.11f		gir	vit
gir	34	23	N9-0.9	gir	20.6	36.4
vit	9	53	C	vit	22.4	39.6
	gir	vit	R407-0.7 /		gir	vit
gir	0	43	N9-0.53f	gir	9.1	16.9
vit	26	54	D	vit	33.9	63.1

cessive allelic replacements in the interacting pair. An alternative is that different sets of interacting alleles happen to be linked, causing reduction of associated RAPD markers with a symmetrical appearance.

The most unexpected result was the complete absence of gg combination in interaction D (involving markers on linkage groups I and III). This pattern requires explanation because it is completely unexpected based upon the standard gene incompatibility models. These associated g alleles clearly do not cause lethality in the *N. giraulti* nuclear environment. The pattern cannot easily be explained by invoking a missing third interacting nuclear gene expressed in F_2 males, since such an interaction would not result in complete absence of the gg genotype. Nuclear-cytoplasmic incompatibility could be involved, but would require the incompatibility to be specific to the hybrid environment, since the regions are compatible with v cytoplasm otherwise. A likely explanation for the pattern is a dominant incompatibility between a maternal effect locus and two zygotic g loci linked to the respective RAPD markers. Because F_1 mothers are v/g at the maternal locus, if the v allele has a dominant lethal interaction with the two zygotic g alleles, then the gg genotype will be absent among adult F_2 males. Maternal effect-zygotic lethal interactions have been previously described in Drosophila hybrids (Sawamura 1996). Follow-up experiments will be necessary to determine the genetic basis of the effect.

As mentioned before, more complex interactions than the four detected are likely to be occurring between the *N. vitripennis* and *N. giraulti* genomes. The conservative statistical approach used excluded many significant effects that did not meet the standards of significance once the Bonferroni correction was applied. However, this correction assumed that all 3252 comparisons were independent. In addition, three-way and more complex interactions were not investigated by this method. However, the analysis has already provided a set of putative hybrid incompatibilities requiring further investigation. An important question concerns whether these particular hybrid incompatibilities are expressed specifically in the hybrid genetic background (*i.e.*, whether the incompatibilities are sensitive to the general disruptions associated with hybrid genomes—"hybrid sickness") or whether the lethal interactions are specific to the set of interacting loci. In the latter case, we should be able to introgress the lethal interaction into either species genetic background for more detailed analysis.

Although the Nasonia species complex seems to be very young it has had enough time to develop a complex web of negative interacting nuclear genes and additional nucleo-cytoplasmic interactions leading to the observed severe hybrid breakdown. If the isolation of the two Nasonia species was maintained by infection with different Wolbachia strains (Breeuwer and Werren 1990; Bordenstein and Werren 1997) then we must assume that the negative interactions leading to the postzygotic hybrid breakdown have evolved purely as an accumulation of new incompatible mutations during the time of isolation and were not due to selection for isolation mechanism in both species. This would be in accordance with the basic Dobzhansky-Muller theory of F_2 hybrid breakdown (Dobzhansky 1937; Muller 1942).

Overall we have demonstrated that the approach of using an interspecific cross as the basis for a linkage map is a fast and efficient way to study the genetic basis of reproductive barriers between closely related species. The approach is particularly promising for haplodiploids, where genetic incompatibilities can be uncovered in the haploid males without the added complexity of their dominance interactions with other alleles.

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