DOI: 10.1534/genetics.107.080523

Cytonuclear Genic Incompatibilities Cause Increased Mortality in Male F_2 Hybrids of *Nasonia girault*i and *N. vitripennis*

Oliver Niehuis,[1] Andrea K. Judson and Jürgen Gadau

School of Life Sciences, Arizona State University, Tempe, Arizona 85287-4501

Manuscript received August 14, 2007
Accepted for publication November 10, 2007

ABSTRACT

The haplodiploid wasp genus Nasonia is a promising model for studying the evolution of genic incompatibilities due to the existence of interfertile species and haploid males. The latter allows for significantly reducing the sample size required to detect and map recessive dysfunctional genic interactions. We exploited these features to study the genetics of intrinsic hybrid inviability in male F_2 hybrids of *Nasonia girault*i and *N. vitripennis*. Analyzing marker segregation in 225 hybrid embryos, we inferred a linkage map with 38 framework markers. The markers were tested for marker transmission ratio distortion (MTRD) and interchromosomal linkage disequilibrium in populations of embryonic and adult hybrids. We found evidence for four transmission ratio distorting loci (TRDL). Three TRDL showed a deficit of the *N. girault*i allele in hybrids with *N. vitripennis* cytoplasm. A separate TRDL exhibited a deficiency of the *N. vitripennis* allele in hybrids with *N. girault*i cytoplasm. We ascribe the observed MTRD in adult hybrids to cytonuclear genic incompatibilities causing differential mortality during development since hybrid embryos did not show MTRD. The identified cytonuclear genic incompatibilities in F_2 hybrids with *N. vitripennis* cytoplasm account for most of the intrinsic hybrid inviability in this cross. The high mortality rate in F_2 hybrids with *N. girault*i cytoplasm cannot be explained by the single identified TRDL alone, however.

UNDERSTANDING the genetic basis of reproductive barriers is one of the major challenges in evolutionary genetics and in speciation genetics in particular (Coyne and Orr 2004). Two frequently observed reproductive barriers that isolate species are intrinsic hybrid inviability and sterility. They can be caused by differences in ploidy levels, chromosomal organization, infection by endosymbionts, and genic incompatibilities (*e.g.*, Stebbins 1950; Werren 1998; Johnson 2000; Delneri *et al.* 2003). So far, only a few studies have identified genes that are incompatible in hybrids and provided insights to how negative genic interactions account for hybrid inviability and sterility (*e.g.*, Wittbrodt *et al.* 1989; Schartl *et al.* 1994; Malitschek *et al.* 1995; Ting *et al.* 1998; Barbash *et al.* 2003; Presgraves *et al.* 2003; Brideau *et al.* 2006). In general, however, little is known about the nature of genic incompatibilities that cause intrinsic postzygotic reproductive isolation (Hutter 1997; Coyne and Orr 2004; Orr *et al.* 2004; Orr 2005). Three problems may account for this lack of knowledge: the difficulty of carrying out genetics between populations that are reproductively isolated (Lewontin 1974; Orr *et al.* 2004; Orr 2005), the complexity of genic incompatibilities that frequently involve more than just two genes (*e.g.*, Muller 1942; Dobzhansky 1975; Cabot *et al.* 1994; Orr and Irving 2001), and the fact that genic incompatibilities tend to be recessive (Muller 1942; Tao and Hartl 2003; see also Coyne and Orr 2004).

The parasitic wasp genus Nasonia has two particular features that allow us to overcome these obstacles and facilitate speciation genetic studies: a haplodiploid sex determination and the ease with which its species can be crossed in the laboratory (Whiting 1967; Skinner and Werren 1980; Breeuwer and Werren 1990; see also Beukeboom and Desplan 2003). In arrhenotokous species, females develop from fertilized eggs and are diploid whereas males develop parthenogenetically from unfertilized eggs and are haploid. Since there are no intralocus interactions in haploid males, alleles can easily be tested individually for their phenotypic effect in this sex. This significantly reduces the population sizes required to detect and map recessive alleles and epistatic loci in haploid F_2 hybrid males (Gadau *et al.* 1999, 2002; Weston *et al.* 1999; Wilfert *et al.* 2007). The genetics of genic incompatibilities can easily be explored in the genus Nasonia, since interspecific F_1 hybrid females are viable and fertile while a certain percentage of their F_2 hybrid male offspring are not (Breeuwer and Werren 1995).

Reproductive isolation between species of Nasonia (Darling and Werren 1990; Campbell *et al.* 1993) has been studied extensively. Under natural conditions,

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. EF638382–EF638434.

[1]*Corresponding author:* School of Life Sciences, Arizona State University, P.O. Box 874501, Tempe, AZ 85287-4501.
E-mail: oliver.niehuis@gmail.com

gene flow is impeded due to the infection of each Nasonia species with different strains of endosymbiotic Wolbachia bacteria (Breeuwer and Werren 1990; Bordenstein *et al.* 2001). These Rickettsia-like bacteria are maternally transmitted in the cytoplasm and are known to alter host reproduction (Werren 1997). In Nasonia, Wolbachia causes cytoplasmic incompatibility (Breeuwer and Werren 1990). Since nearly all individuals of Nasonia are doubly infected with two species-specific Wolbachia strains, the cytoplasmic incompatibility is bidirectional (Breeuwer and Werren 1990, 1993; Bordenstein and Werren 1999). All Nasonia species hybridize, however, once they are cured of their Wolbachia endosymbionts. Interspecific sexual isolation occurs, but it is asymmetrical and incomplete (Drapeau and Werren 1999; Bordenstein *et al.* 2000; Velthuis *et al.* 2005). While F_1 hybrid females are viable and fecund, haploid F_2 hybrids from crosses between *Nasonia vitripennis* and *N. giraulti* or *N. longicornis* suffer from increased mortality (Breeuwer and Werren 1995; C. Pietsch, L. Beukeboom and J. Gadau, unpublished results).

Hybrid breakdown, the increased sterility and mortality in F_2 and later hybrid generations, is thought to be caused by the recombination of two differentially coadapted parental genomes (Stebbins 1950; Coyne and Orr 2004). In Nasonia, hybrid breakdown was first investigated by Breeuwer and Werren (1995) in F_2 hybrids of *N. giraulti* and *N. vitripennis* using the inbred strains RV2T and AsymC. F_2 hybrid males in this cross suffer from increased mortality during larval development. F_2 hybrid males with *N. giraulti* cytoplasm experience additional mortality during the pupal stage. Diploid F_2 hybrid females obtained by backcrossing the F_1 hybrid females with males of either species suffer only marginally from hybrid breakdown, suggesting that the underlying genic incompatibilities are recessive and can be amended by adding a complete genome complement. Breeuwer and Werren (1995) suggested that both nuclear–nuclear and nuclear–cytoplasmic (=cytonuclear) genic incompatibilities are involved in *N. giraulti* × *N. vitripennis* F_2 hybrid breakdown.

To map the nuclear–nuclear genic incompatibilities that had been hypothesized by Breeuwer and Werren (1995), Gadau *et al.* (1999) searched for significant deviations from expected recombinant and nonrecombinant genotypes between markers from different linkage groups (LGs) in haploid *N. giraulti* × *N. vitripennis* F_2 hybrids with *N. vitripennis* cytoplasm. However, the authors chose to use a *N. giraulti* strain with *N. vitripennis* cytoplasm (*i.e.*, R16A) for their cross experiment instead of the *N. giraulti* inbred strain RV2T with the idea of eliminating the impact of cytonuclear genic incompatibilities on their analysis. Interestingly, many markers in their study showed a significant bias toward *N. vitripennis* alleles (*i.e.*, marker transmission ratio distortion, or MTRD), despite the fact that the *N. giraulti* nuclear genome was already introgressed in *N. vitripennis* cytoplasm and showed no incompatibilities within the R16A strain. The cause of the observed bias—potentially cytonuclear genic incompatibility due to a deficient introgression of the *N. giraulti* nuclear genome in the *N. vitripennis* cytoplasm—remained to be investigated.

MTRD, the preferential inheritance of one parental allele, is frequently observed in interspecific crosses (*e.g.*, Hall and Willis 2005; Moyle and Graham 2006 and references therein), and several mechanisms that can cause a bias in the recovery rate of parental alleles are known. Selfish genetic elements, for example, are known to transmit themselves at the expense of their homologs (Lyttle 1991; Hurst 1993; Hurst and Werren 2001). Well-studied examples are the *Segregation distorter* in Drosophila (Ganetsky 1977; Wu *et al.* 1988; Merrill *et al.* 1999) and the *t* haplotypes in mice (Silver 1993). The underlying process that causes the corresponding alleles to be overrepresented in the gametes is referred to as meiotic drive, although the mechanisms of this process can differ significantly. Meiotic drive has been reported to drive the evolution of reproductive isolation (*e.g.*, Merçot *et al.* 1995; Cazemajor *et al.* 1997; Montchamp-Moreau and Joly 1997; Tao *et al.* 2001; Fishmann and Willis 2005; Orr and Irving 2005). MTRD can also occur because of postzygotic viability differences due to an incompatibility of nuclear alleles with cytoplasmic factors (*e.g.*, mitochondrial-encoded genes; Fishmann *et al.* 2001; Fishmann and Willis 2006). In most cases, however, the mechanism causing MTRD is unknown (Pardo-Manuel De Villena *et al.* 2000).

In this study, we investigate the occurrence and cause of MTRD in F_2 hybrid males of *N. giraulti* and *N. vitripennis*, considering both directions of the cross. We address the following questions:

i. Is MTRD also observed in crosses between the inbred strains RV2X(U) and AsymCX of *N. giraulti* and *N. vitripennis*, respectively? These strains are descended from AsymC and RV2T, which had been studied by Breeuwer and Werren (1995), and are assumed to be genetically identical with them.
ii. Does MTRD differ between the reciprocal crosses?
iii. How many transmission ratio distorting loci (TRDL) have to be assumed to explain the extent of MTRD in F_2 hybrid males that differ in their cytoplasm, and where in the genome are they located?
iv. How much of the observed mortality can the TRDL explain?
v. Which TRDL allele is more frequently transmitted to adult F_2 hybrid males with a given cytoplasm? For example, is the *N. vitripennis* allele of a TRDL more frequently found in adult F_2 hybrids males with *N. vitripennis* cytoplasm than the *N. giraulti* allele?
vi. Do male F_2 hybrid embryos already exhibit MTRD?

By answering these questions, we have taken the first step in identifying the genes that cause MTRD in Nasonia. The recently sequenced Nasonia genome (http://www.hgsc.bcm.tmc.edu/projects/nasonia/) provides the means for continuing this line of research and makes this a goal within reach.

MATERIALS AND METHODS

Stocks: We used the Nasonia strains AsymCX and RV2X(U) for the cross experiments. AsymCX is derived from the Wolbachia-infected *N. vitripennis* wild-type strain LBii (LabII); its geographical origin is Leiden, The Netherlands (Breeuwer and Werren 1995). RV2X(U) is descended from the Wolbachia-infected *N. girault*i wild-type strain RV2, which had been collected in Rochester, New York (Breeuwer and Werren 1995). We chose these two strains because they are highly inbred (*i.e.*, homozygous for every marker tested so far), antibiotically cured of their endosymbiotic Wolbachia bacteria (allowing the formation of interspecific F_1 hybrids), and were used for the genome sequencing (Breeuwer and Werren 1990, 1993; Breeuwer *et al.* 1992; http://www.hgsc.bcm.tmc.edu/projects/nasonia/). The two Nasonia laboratory strains and their hybrids were cultured in an incubator on pupae of the flesh fly (*Sarcophaga bullata*) at 25° and with permanent light.

Cross experiments: We began the cross experiments by collecting males and females of the two strains AsymCX and RV2X(U) as virgin pupae and raised each individual in a separate vial. Once the wasps had eclosed, we provided them with a 3:1 solution of honey to water and kept them in the incubator for another 2 days. We then placed each female together with a single male of the other strain in a small glass tube (12 × 75 mm; Fischer Scientific, Fair Lawn, NJ). After 24 hr, each pair was provided with two host puparia. We collected the F_1 hybrid females as virgin pupae 10 days later and placed them in separate vials. Their male siblings were not hybrids, since they developed from unfertilized eggs. Eclosed F_1 hybrid females were fed with a 3:1 solution of honey to water and provided with a host puparium 2 days later. Since the F_1 hybrid females were virgins, they laid unfertilized eggs, which developed into haploid (hemizygote) F_2 hybrid males. We opened a fraction of parasitized host puparia after 12–16 hr and, using a sterile pin, removed 120 F_2 hybrid embryos each from the cross AsymCX (♀) × RV2X(U) (♂) and the reciprocal cross AsymCX (♂) × RV2X(U) (♀). Each embryo was transferred into a separate 0.2-μl PCR tube and stored at −70° for subsequent procedures. The remaining host puparia were left intact for 10 days. We then carefully opened them with a pair of forceps to facilitate the emergence of the adult wasps, because F_2 hybrid males are not capable of opening their host puparium themselves (our personal observation). Within 36 hr after their eclosion, we collected 120 adult F_2 hybrid males each from the cross AsymCX (♀) × RV2X(U) (♂) and the reciprocal cross AsymCX (♂) × RV2X(U) (♀). The adult wasps were transferred into 95% ethanol and stored at −70° for subsequent procedures. To verify TRDL (see below) predicted from analyzing the two populations of adult F_2 hybrid males, we repeated the cross experiments and collected an additional 120 adult F_2 hybrid males from each cross within 36 hr after they hatched. These hybrids were also stored in 95% ethanol at −70°.

Molecular procedures: DNA from adult wasps was extracted using a Chelex extraction protocol described by Niehuis *et al.* (2007). Due to the small amount of genomic DNA in early F_2 hybrid embryos, DNA was preamplified for use in subsequent procedures using the GenomiPhi DNA amplification kit (Amersham Biosciences, Piscataway, NJ). Each embryo was first homogenized with the aid of a sealed sterile 10-μl pipette tip in 1 μl alkaline lysis solution (400 mm KOH, 100 mm DTT, 10 mm EDTA). After 10 min incubation, we added 1 μl neutralization buffer (400 mm HCl, 600 mm Tris–HCl, pH 6.0) to the lysate and the lysate was briefly mixed well. Each sample tube was then filled up to 10 μl with sample buffer (included in the kit) and heated for 3 min at 95°. After the samples were cooled to 4° on ice, we amplified the template DNA with the GenomiPhi kit reaction premix following the manufacturer's recommendations. The amplification products were subsequently purified using a standard ethanol precipitation protocol. Quantity and quality of Chelex DNA extracts and of the GenomiPhi amplified DNA were assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Marker segregation data were collected using polymerase chain reaction (PCR)-based methods and consisted of three types of molecular genetic markers for sampling the nuclear genome: length polymorphic (LP) markers, present/absent (P/A) markers, and single nucleotide polymorphism (SNP) markers. Most of the LP markers and all P/A markers were adopted from Pietsch *et al.* (2004), Rütten *et al.* (2004), Pietsch (2005), and Niehuis *et al.* (2007); markers published by Rütten *et al.* (2004) were chromosomally anchored. Additional LP markers had been reported by Gadau *et al.* (2008), but lacked a formal characterization so far (see appendix). SNP markers were adopted from Niehuis *et al.* (2007) and Gadau *et al.* (2008) (see appendix).

The genotype of LP and P/A markers was inferred by separating their PCR products on a denaturing polyacrylamide gel. The PCRs were performed in 12.5-μl volumes [1× colorless GoTaq reaction buffer, 0.625 units GoTaq polymerase (Promega, Madison, WI), 1.6 mm dNTP mix, 0.4 μm of each primer, 10 ng DNA] using an Eppendorf epGradient Mastercycler (Eppendorf, Hamburg, Germany). To facilitate the detection of the PCR products, we used fluorescently (IR700/800) labeled oligonucleotide primers. The applied PCR temperature profiles are given in the appendix. All fluorescently labeled PCR products were separated on a LI-COR 4300 DNA analysis system (LI-COR, Lincoln, NE) and analyzed using the SAGA Generation 2 software (LI-COR). SNP markers were genotyped with the recently described Ecotilling technique (Comai *et al.* 2004), applying the protocol given by Niehuis *et al.* (2007).

The genotype of the mitochondria in hybrids was inferred by sequencing a 385-bp-long fragment of the mitochondrial-encoded gene Cytochome oxidase subunit I (COI). Since all F_1 hybrid females in our cross experiments had laid unfertilized eggs, we genotyped only one representative F_2 hybrid male from each F_1 hybrid female's offspring. The COI fragment was amplified via PCR [25 μl volume (1× colorless GoTaq reaction buffer, 0.625 units GoTaq polymerase (Promega), 1.6 mm dNTP mix, 0.4 μm of each primer (see appendix), 20 ng DNA] using a PTC-100 Peltier Thermal Cycler (MJ Research, Watertown, MA). The applied PCR temperature profile is given in the appendix. After checking the PCR products on 1.5% agarose mini-gels, we enzymatically cleaned them up using the ExoSAP-IT kit (USB, Cleveland). Both strands of the DNA fragments were then sequenced in the Arizona State University sequencing facility on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). We assembled the complementary strands with the Sequencher 4.6 software (Gene Codes, Ann Arbor, MI) and aligned the contigs in BioEdit 7.0.5.3 (Hall 1999). The sequences are deposited in GenBank under the accession nos. EF638404–EF638434.

Linkage map reconstruction: Marker segregation data were studied with the program MultiPoint (http://www.multiqtl.com), which infers marker order and map distances using a multipoint likelihood approach (Mester *et al.* 2003a,b, 2004). To minimize the effect of negatively interacting loci causing mortality on the linkage map reconstruction due to a phenomenon commonly referred to as quasi- or pseudolinkage (Korol *et al.* 1989; Lorieux *et al.* 1995a,b; Liu 1998; Weston *et al.* 1999; Peng *et al.* 2000), we considered only F_2 hybrid embryos for linkage analysis. We first evaluated the mapping population ($N = 225$) by analyzing markers for missing information and MTRD. We subsequently searched for putative sets of linkage groups by calculating pairwise recombination fractions (rf_s) for all pairs of markers using a maximum-likelihood estimation procedure as implemented in the software. We chose $rf_s = 0.35$ as a reasonable threshold level as it prevented fusion of anchored markers from different chromosomes or disruption of anchored markers on single chromosomes. The optimal marker order in individual linkage groups was found by minimizing the total length of the multi-locus map while iteratively testing for marker-order stability using a Jackknife approach (sampling 90% of the total population, 1000 iterations) and successively removing markers with uncertain position. Markers violating a monotonic increase of rf_s along linkage groups were finally deleted by applying the control for monotony function. After a consistent linear order of markers was reached, we calculated map distances in centimorgans from the recombination fractions using Haldane's mapping function (Haldane 1919). We refrained from using Kosambi's mapping function (Kosambi 1944), since the Bayesian approach that we used to map TRDL (see below) assumes a simple noninterference crossover model (*i.e.*, Haldane's mapping function; Vogl and Xu 2000).

Genome length and coverage estimation: The length of individual linkage groups was estimated employing "method 4" described by Chakravarti *et al.* (1991). Thus, assuming a uniform distribution of markers on the linkage groups, the map distance between the most distal markers on each linkage group was inflated by multiplying the factor $(m_i + 1)/(m_i - 1)$, where m_i is the number of markers on the linkage group (Chakravarti *et al.* 1991). We tested for a uniform marker distribution with the Kolmogorov–Smirnov (K–S) goodness-of-fit test for continuous data (Zar 1999) and employed the correction for small sample sizes expounded by Harter *et al.* (1984) and Khamis (1990, 1993). The total genome length, L, was calculated as sum of the estimated lengths of all linkage groups. To estimate the genome coverage, we considered two approaches: (i) applying the formula $c = 1 - e^{-2nd/L}$, provided by Lange and Boehnke (1982), with c being the fraction of the genome within d cM of a marker on the linkage map and n being the total number of markers on the linkage map; it assumes that markers are randomly distributed on the linkage map; (ii) dividing the estimated total genome length through the summed lengths of all linkage groups. To test for a random distribution of markers on the linkage map, we compared the distribution of marker intervals of consecutive markers along linkage groups with the null hypothesis that they follow a normal distribution applying the Kolmogorov–Smirnov one-sample test and Lilliefors' significance correction (Lilliefors 1967). We calculated the average marker spacing by dividing the summed lengths of all linkage groups by the number of marker intervals.

Analysis of marker segregation ratios: Markers in the data set were first explored in Microsoft Excel 2003 (Microsoft, Redmond, WA) with the χ^2 test for goodness of fit (Zar 1999) to the expected 1:1 segregation ratio of parental alleles (d.f. = 1) and applying Yates' correction for continuity (Yates 1934). We applied a Bonferroni correction to lower the number of false positives by dividing $\alpha = 0.01$ by the number of linkage groups (*i.e.*, five). Our criterion for rejecting the null hypothesis (*i.e.*, no MTRD) was thus $P < 0.002$. We further applied a Bayesian multipoint mapping approach developed by Vogl and Xu (2000) and implemented in the software ANITA (provided by Claus Vogl) to estimate the number, location, and effect of TRDL on each linkage group. The maximum number of TRDL per linkage group was set to four and the position of analyzed markers predefined by adopting the inferred linkage map after removing redundant markers (*i.e.*, NvC1-22 and Nv-23; these markers map at the same location as EF-1α F1 and Nv-36, respectively). For each linkage group, we ran one Markov chain with random start parameters and 21,000 iterations. The first 1000 iterations were conservatively discarded to account for a possible burn-in period of the Markov chain. The remaining samples of the Markov chain were subsequently analyzed in Microsoft Excel 2003 and SPSS 14.0 (SPSS, Chicago). To measure the change of the odds between the prior and posterior probability for the number of TRDL on a given chromosome, we calculated Bayes factors (Lavine and Schervish 1999). The density distributions of the posterior probability for position and effect of TRDL were approximated by grouping sample values in successive bins of 1 cM and of 1% *N. vitripennis* alleles, respectively. For specifying intervals, we used a square bracket notation. Specifically, $[x,y[$ indicates an interval ranging from x to y, including the value x and excluding the value y. As an estimate of the most likely position and effect of TRDL, we took the mode of the estimated posterior probability distributions. Selected markers close to hypothesized TRDL were finally tested for distorted segregation ratios in a second population with the same cytoplasm as the one in which the effect was initially observed. Segregation data were further explored for negative nuclear–nuclear and nuclear–nuclear–cytoplasmic (*i.e.*, between a cytoplasmic and two nuclear loci) genic interactions by testing for deviations between the expected and the observed frequency of recombinant and nonrecombinant genotypes between markers from different linkage groups (see also Gadau *et al.* 1999; Sawamura *et al.* 2006), assuming an independent assortment of markers on different chromosomes. The tests were performed with the help of a Perl script using the χ^2 test for goodness of fit (d.f. = 1). To lower the number of false positives, we applied a Bonferroni correction by dividing $\alpha = 0.01$ by the number of possible chromosome pairs (*i.e.*, 10). Our criterion for rejecting the null hypothesis was thus $P < 0.001$. As for the hypothesized TRDL, selected markers close to possible negatively interacting nuclear loci were subsequently tested for linkage disequilibrium in a second population with the same cytoplasm as the one in which the effect was initially observed.

RESULTS

Mapping populations: We obtained six mapping populations from our cross experiments. Three of them had *N. giraulti* cytoplasm (hereafter indicated by [*g*]) and three had *N. vitripennis* cytoplasm (hereafter indicated by [*v*]). The genotype of the cytoplasm was confirmed by sequencing a fragment of the mitochondrial-encoded gene COI in representative F_2 hybrids ($N = 31$). For each direction of the *N. giraulti* × *N. vitripennis* cross, there were thus two populations of adult F_2 hybrid males, one for identifying TRDL ($N_{[g]} = 120$, $N_{[v]} = 116$) and one for verifying them ($N_{[g]} = 120$, $N_{[v]} = 120$), as well as an additional population with <25-hr-old F_2 hybrid male embryos ($N_{[g]} = 112$, $N_{[v]} = 113$).

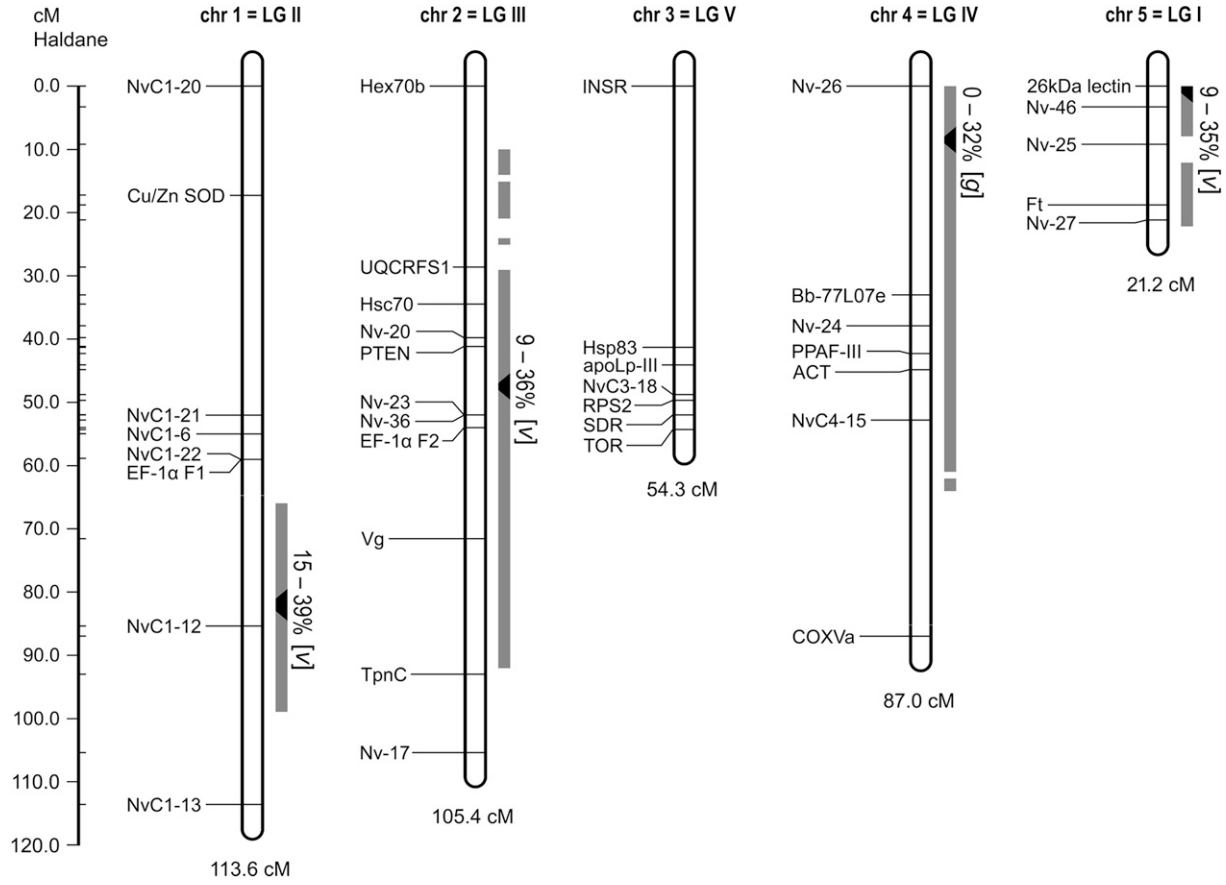

FIGURE 1.—Linkage map inferred from marker segregation data in a *N. giraulti* × *N. vitripennis* F_2 hybrid population of male embryos ($N = 225$). Recombination distances are shown in Haldane centimorgans. Bars near chromosomes specify the 95% confidence limits for the position of predicted TRDL; arrowheads point to the region with the highest posterior probability for the position of TRDL. Percentages indicate the estimated mortality caused by each TRDL in a population of F_2 hybrid males with [*g*] = *N. giraulti* and [*v*] = *N. vitripennis* cytoplasm, respectively.

Linkage map: To exclude pseudolinkage problems, we considered only segregation data from hybrid embryos for the generation of a linkage map. The linkage analysis is based on segregation data of 47 markers in 225 haploid F_2 hybrids (*i.e.*, 112 F_2 hybrid embryos with *N. giraulti* cytoplasm plus 113 F_2 hybrid embryos with *N. vitripennis* cytoplasm). The phase of the markers was known from prior screening representatives of each of the two parental highly inbred strains [*i.e.*, RV2X(U), AsymCX]. Nine of the 47 markers were removed from the data set during the mapping process because they significantly reduced marker-order stability and/or violated the monotony criterion.

The inferred *N. giraulti* × *N. vitripennis* linkage map consists of 38 molecular markers (characterized in the APPENDIX), which mapped into five linkage groups. By considering chromosome anchored microsatellite markers (RÜTTEN *et al.* 2004), it was possible to homologize individual LGs with chromosomes (chr) (Figure 1).

Estimated chromosome lengths and genome coverage: The *N. giraulti* × *N. vitripennis* chromosome map spans 381.5 cM (Haldane mapping function). The lengths of the chromosomes are 113.6 cM (chr 1), 105.4 cM (chr 2), 54.3 cM (chr 3), 87.0 cM (chr 4), and 21.2 cM (chr 5), respectively. Since the distribution of the markers on chromosomes 1, 2, 4, and 5 does not significantly differ from a uniform distribution (K–S test, $P > 0.5$), we estimated the chromosome lengths by inflating the observed chromosome lengths with the formula described by CHAKRAVARTI *et al.* (1991) under "method 4." The estimated lengths are 146.1 cM (chr 1), 126.5 cM (chr 2), 116.0 cM (chr 4), and 31.8 cM (chr 5). The most distant markers on each chromosome would accordingly span 78, 83, 75, and 67%, respectively, of its actual length. Applying the same method for chromosome 3 provided an estimated length of 72.4 cM, and the most distant markers on this chromosome would span 75% of its total length. However, the distribution of the markers on chromosome 3 significantly differed from a uniform distribution (K–S test, $P < 0.01$).

The estimated total genome length, *L*, as sum of the estimated lengths of all linkage groups is 492.8 cM. Our *N. giraulti* × *N. vitripennis* chromosome map would consequently span 77% of the total genome, but this estimate includes the questionable value for chromosome 3. We also considered the method proposed by LANGE

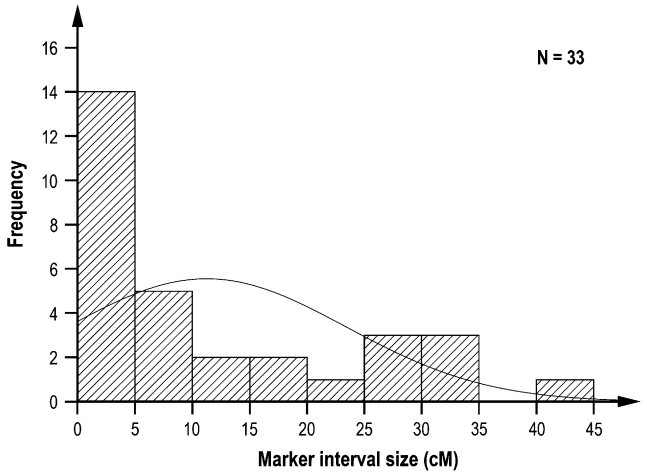

FIGURE 2.—Distribution of the interval size between consecutive markers in the *N. vitripennis* × *N. giraulti* chromosome map. The superimposed graph shows a normal distribution with a mean of 11.7 cM against which the observed interval size distribution was tested (K–S test, $P < 0.01$).

and BOEHNKE (1982) to estimate the genome that is covered within 10 and 20 cM, respectively, of a marker on the map. The corresponding values are $c_{10cM} = 79\%$ and $c_{20cM} = 95\%$. This calculation assumes a random distribution of markers on the chromosome map and an accurate estimate of the total genome length. The distribution of marker intervals of consecutive markers along the linkage groups, however, shows that markers tend to cluster (Figure 2). Compared to an expected average marker spacing of 11.6 cM, there is a disproportionally high frequency of intervals with a length of 0–5 cM (Figure 2). Testing the observed distribution of marker intervals against the null hypothesis that they follow a normal distribution showed this difference as significant (K–S test, $P < 0.01$).

Marker transmission ratio distortion: None of the markers in the two F_2 hybrid populations of male embryos showed a significant bias from a 1:1 Mendelian segregation ratio (χ^2 test; $P > 0.05$). The same was true for adult F_2 hybrid males with *N. giraulti* cytoplasm (χ^2 test; $P > 0.05$). By contrast, 11 of the 38 markers in adult male hybrids with *N. vitripennis* cytoplasm exhibited MTRD (χ^2 test at $P < 0.002$): NvC1-12 (chr 1), Nv-20, Nv-23, Nv-36, EF-1α F2, PTEN, Vg (chr 2), Nv-27, Nv-46, Ft, 26-kDa lectin (chr 5).

The Bayesian analyses of segregation ratios in adult wasps corroborated the existence of TRDL in hybrids with *N. vitripennis* cytoplasm on chromosomes 1, 2, and 5 (Table 1); the posterior probabilities (hereafter abbreviated pP) for these assumptions were 99.5, 97.89, and 97.58%. The pP for no TRDL was >50% for the remaining chromosomes in hybrids with *N. vitripennis* cytoplasm and for all chromosomes in hybrids with *N. giraulti* cytoplasm, except for chromosome 4, in which the pP for the presence of TRDL was 55.09%.

The calculated Bayes factors (Table 1), which are a measure of the change of the odds between the prior and posterior probability for hypotheses, suggested that only one TRDL on each of the four chromosomes mentioned above (*i.e.*, chr 1 [*v*], chr 2 [*v*], chr 4 [*g*], and chr 5 [*v*]) would likely be responsible for the bias of markers. For the three chromosomes in F_2 hybrid males with *N. vitripennis* cytoplasm, this assumption was reasonably well supported as indicated by Bayes factors of 9.7 (chr 1), 6.1 (chr 2), and 8.0 (chr 5). For chromosome 4 in F_2 hybrids with *N. giraulti* cytoplasm, however, the data set changed the odds in favor of one TRDL by only 2.1.

The Bayesian multipoint mapping suggested that the putative TRDL on chromosome 1 is with 95% confidence in the interval [66,99[cM, with the highest pP between [81,83[cM. The obtained density function for the position of the putative TRDL on chromosome 2 was more complex, however, and the 95% confidence interval is discontinuous: [10,14[cM + [15,21[cM + [22,23[cM + [24,25[cM + [29,92[cM. The highest pP was found for the interval [47,48[cM. The putative TRDL on chromosome 4 is with 95% confidence within the two intervals [0,61[cM and [62,64[cM, with the highest pP between [8,9[cM. The putative TRDL on chromosome 5, finally, is with 95% confidence in the interval [0,8[cM + [11,21.2[cM; the highest pP was found for the interval [0,1[cM. The results are summarized in Figure 3A.

We further used the Bayesian mapping approach to estimate the effect of putative TRDL in terms of the *N. vitripennis* allele recovery rate at a TRDL. For the putative TRDL on chromosome 1, the analysis suggested with 95% confidence a recovery rate of *N. vitripennis* between 59 and 82%; the highest pP was between 67 and 68%. For chromosome 2, the recovery rate with 95% confidence at the TRDL is between 55 and 78%; the highest pP is between 63 and 64%. For the TRDL on chromosome 4, the 95% confidence limits for the recovery rate of *N. vitripennis* are 27–51%, with the highest pP between 41 and 42%. The confidence limits for the effect on chromosome 5 are 55–77%, with the highest pP obtained for a recovery rate between 63 and 64%. The results are summarized in Figure 3B.

On the basis of the preceding analysis of MTRD in the first two populations of adult F_2 hybrid males, we selected a total of six markers close to the most likely positions of the four hypothesized TRDL to test their segregation ratios in two independent populations of adult F_2 hybrid males (Figure 3A). Specifically, we chose NvC1-12 (chr 1), Nv-20, Nv-36, Vg (chr 2), and 26-kDa lectin (chr 5) for testing in the second set of F_2 hybrid males with *N. vitripennis* cytoplasm and Nv-26 (chr 4) for testing in the second set of F_2 hybrid males with *N. giraulti* cytoplasm. Since only a limited number of tests had to be performed and because these tests were meant to confirm the existence and direction of the hypothesized TRDL, we applied a conservative Bonferroni correction by dividing $\alpha = 0.01$ by 5 while testing

TABLE 1

Posterior probabilities for the number of TRDL on individual chromosomes in four mapping populations of F_2 hybrids of *N. giraulti* and *N. vitripennis*

Population[a]	Chromosome	Posterior probability[b] for no. of TRDL and corresponding Bayes factors[c]									
		0 (%)	B_{12} (0)	1 (%)	B_{12} (1)	2 (%)	B_{12} (2)	3 (%)	B_{12} (3)	4 (%)	B_{12} (4)
Embryonic F_2 hybrids [*g*]	1	*66.85*	1.3	28.82	0.9	3.88	0.5	0.45	0.4	0.01	0.1
($N = 112$)	2	*87.70*	4.6	10.96	0.3	1.21	0.1	0.13	0.1	0.00	0.0
	3	*88.87*	5.2	9.62	0.2	1.38	0.2	0.13	0.1	0.01	0.1
	4	*83.05*	3.2	14.64	0.4	2.09	0.3	0.21	0.2	0.02	0.1
	5	*86.15*	4.0	11.67	0.3	1.97	0.2	0.22	0.2	0.01	0.1
Adult F_2 hybrids [*g*]	1	*73.24*	1.8	18.38	0.5	8.75	1.2	1.53	1.2	0.12	0.8
($N = 120$)	2	*85.20*	3.7	12.79	0.3	1.71	0.2	0.29	0.2	0.02	0.1
	3	*89.08*	5.3	9.43	0.2	1.28	0.2	0.21	0.2	0.02	0.1
	4	*44.93*	0.5	*48.15*	2.1	6.24	0.8	0.66	0.5	0.04	0.3
	5	*90.30*	6.0	8.43	0.2	1.09	0.1	0.18	0.1	0.01	0.1
Embryonic F_2 hybrids [*v*]	1	*87.31*	4.5	11.23	0.3	1.32	0.2	0.15	0.1	0.01	0.1
($N = 113$)	2	*88.28*	4.9	10.66	0.3	0.97	0.1	0.10	0.1	0.00	0.0
	3	*88.09*	4.8	10.24	0.3	1.49	0.2	0.17	0.1	0.03	0.2
	4	*84.34*	3.5	13.96	0.4	1.49	0.2	0.21	0.2	0.01	0.1
	5	*54.35*	0.8	38.62	1.4	6.20	0.8	0.81	0.6	0.03	0.2
Adult F_2 hybrids [*v*]	1	0.50	0.0	*80.86*	9.7	16.48	2.4	2.03	1.6	0.13	0.8
($N = 116$)	2	2.11	0.0	*72.79*	6.1	21.75	3.4	3.21	2.6	0.15	0.9
	3	*78.13*	2.3	15.09	0.4	5.65	0.7	1.01	0.8	0.13	0.8
	4	*80.71*	2.7	17.20	0.5	1.88	0.2	0.20	0.2	0.03	0.2
	5	2.43	0.0	*77.70*	8.0	16.45	2.4	3.19	2.6	0.24	1.5

[a] Genotype of cytoplasm is indicated by [*g*] for *N. giraulti* and [*v*] for *N. vitripennis*, respectively.

[b] Posterior probability values calculated from 20,000 Markov chain Monte Carlo samples taken from the stationary phase and assuming a Poisson prior ($\lambda = 0.5$) for the no. of TRDL. Highest posterior probabilities are in italics.

[c] Bayes factors (B_{12}) indicate the change of the odds between the prior and posterior probability for the tested hypotheses.

for a segregation ratio distortion of the five markers in F_2 hybrid males with *N. vitripennis* cytoplasm. The criterion for rejecting the null hypothesis accordingly was $P < 0.002$. The criterion for rejecting the null hypothesis in F_2 hybrid males with *N. giraulti* cytoplasm was $P < 0.01$. The results of the tests for MTRD are illustrated in Figure 4. As predicted, we found a significant distortion in at least one of the selected markers on each of the four chromosomes. Furthermore, the specific direction of the distortions corresponded exactly with those predicted from the first two mapping populations. By contrast, none of the deviations from the expected 1:1 segregation ratios in these markers was significant in embryos, and in two instances (NvC1-12 and 26-kDa lectin) the specific direction of the observed bias in embryos is opposed to that found in adult wasps.

Linkage disequilibrium: A comparison between the expected and observed frequency of recombinant and nonrecombinant genotypes between markers from different chromosomes in the first set of adult F_2 hybrids indicated significant deviations in five pairs of markers in F_2 males with *N. vitripennis* cytoplasm (χ^2 test at $P < 0.001$): RPS2/Nv-24, RPS2/ACT, SDR/Nv-24, SDR/Bb-77L07e, and SDR/ACT. However, none of these pairs was found significantly biased in the second population of adult F_2 hybrid males with *N. vitripennis* cytoplasm (χ^2 test, $P > 0.3$). We further compared the expected and the observed frequency of recombinant and nonrecombinant genotypes between markers from different linkage groups in the F_2 hybrid populations of adult males combined, but none of the tests were significant (χ^2 test at $P < 0.001$).

DISCUSSION

This study investigated the genetic basis of F_2 hybrid breakdown in the parasitic wasp genus Nasonia. Hybrid breakdown contributes to the integrity of diverged genomes from closely related and occasionally hybridizing species and thus may play an important role in speciation. The results in this investigation revealed that cytonuclear genic incompatibilities contribute significantly to hybrid breakdown in male F_2 hybrids of *N. giraulti* and *N. vitripennis*. MTRD, the preferential inheritance of one parental allele, was found to be a direct consequence of cytonuclear genic incompatibility. Not all cytonuclear genic incompatibilities in F_2 hybrids of *N. giraulti* and *N. vitripennis* appear to manifest in MTRD, however.

MTRD in male F_2 hybrids of *N. giraulti* and *N. vitripennis* was first reported by Gadau *et al.* (1999). Since the authors considered only one direction of the cross (*i.e.*, *N. giraulti* [*v*] ♀ × *N. vitripennis* ♂), it remained unclear whether or not the phenomenon is restricted to

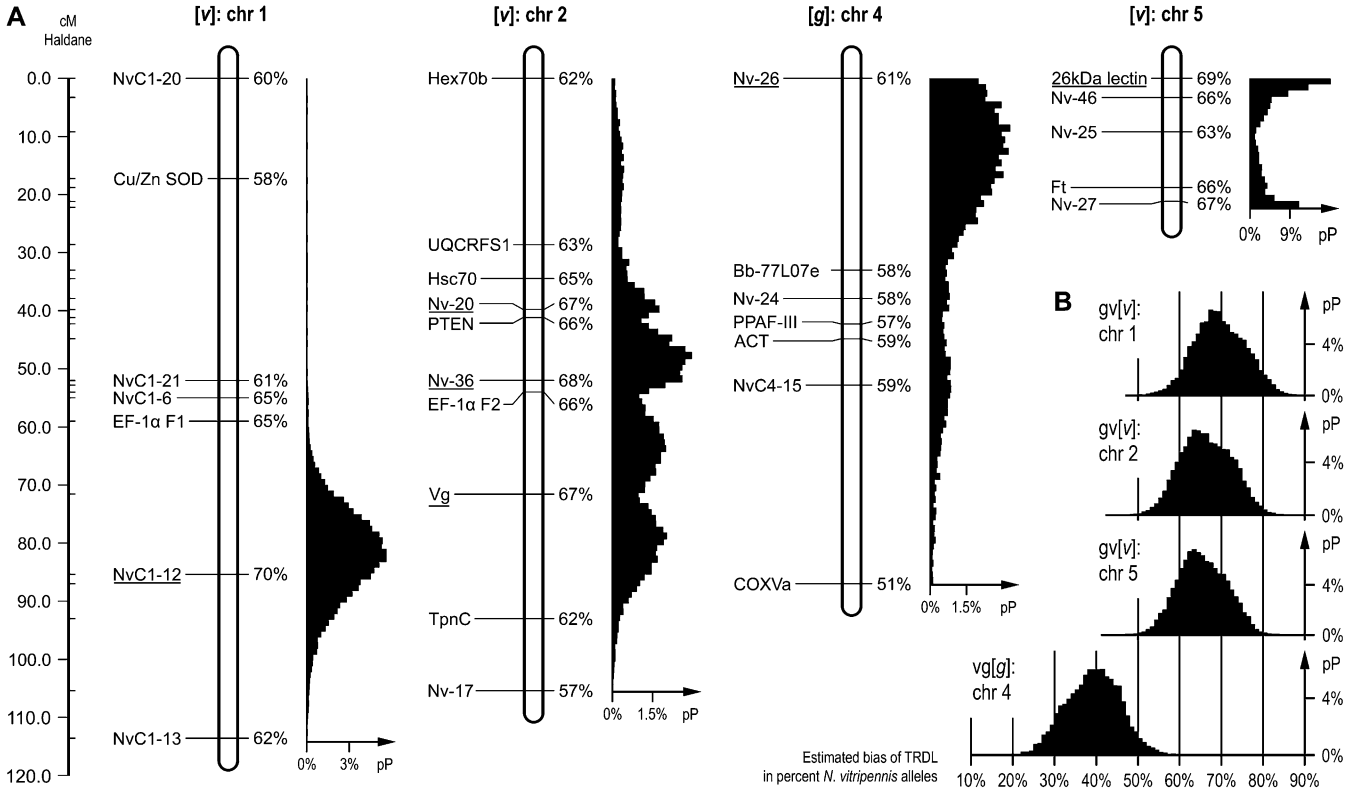

FIGURE 3.—Density distributions of the posterior probability for (A) position and (B) effect of TRDL on chromosomes in adult male F_2 hybrids of *N. giraulti* and *N. vitripennis*. Each distribution was calculated from 20,000 Markov chain Monte Carlo samples taken from the stationary phase and assuming a Poisson prior ($\lambda = 0.5$) for the number of TRDL. Shown are density distributions for one TRDL on each of the depicted chromosomes; the posterior probabilities for these assumptions were 80.86, 72.79, 48.15, and 77.70% (see Table 1). The genotype of the cytoplasm of the population, in which marker transmission ratio distortion was seen, is indicated by [*g*] = *N. giraulti* and [*v*] = *N. vitripennis*, respectively. Underlined markers were tested for segregation ratio distortion in a second population of adult F_2 hybrid males to confirm the TRDL (see Figure 4).

F_2 hybrids with *N. vitripennis* cytoplasm. GADAU *et al.* (1999) furthermore used a *N. giraulti* introgression strain with *N. vitripennis* cytoplasm (R16A) to conduct their cross experiment. It was therefore possible that MTRD occurs exclusively in combination with the R16A strain. Our data revealed that this is not the case: MTRD is also observed in adult male F_2 hybrids when the *N. vitripennis* and *N. giraulti* inbred strains AsymCX and RV2X(U) are crossed. Also, MTRD is not restricted to hybrids with *N. vitripennis* cytoplasm but it is found in adult F_2 hybrid males with *N. giraulti* cytoplasm as well (Figures 3 and 4).

The estimated number and position of TRDL required to explain the biased recovery rate of parental alleles of markers in the investigated adult F_2 hybrid males differed significantly between the reciprocal crosses. While we found evidence for only one TRDL on chromosome 4 in hybrids with *N. giraulti* cytoplasm, at least three TRDL, one each on chromosomes 1, 2, and 5, had to be assumed in hybrids with *N. vitripennis* cytoplasm. Since we used highly inbred strains to conduct the cross experiments, the F_1 hybrid females from reciprocal crosses had identical nuclear genomes. There are no sex chromosomes in haplodiploid wasps. The reciprocal crosses thus differed only in their cytoplasm. The significant differences in the recovery rate of parental alleles in the reciprocal crosses therefore indicate that factors in the cytoplasm are pivotal for the MTRD in the adult F_2 hybrid males.

In contrast to the adult F_2 hybrid males, their embryos did not exhibit significant MTRD. This excludes meiotic drive as an explanation for the observed MTRD in the adult hybrid wasps since this process leads to an overrepresentation of certain alleles in the gametes and therefore should already be detectable in the embryos. The fact that we found MTRD in adult F_2 hybrid males but not in their embryos suggests that the MTRD is the result of postzygotic viability differences of the F_2 hybrid males contingent upon their cytoplasm and nuclear TRDL genotypes. All four identified TRDL exhibited a deficit of the paternal allele. This result is consistent with an incompatibility between paternal TRDL alleles and maternal factors in the cytoplasm. A maternal effect seems unlikely since the F_1 hybrid females from the reciprocal crosses had identical nuclear genomes, and maternal-effect genes generally affect early developmental processes in the offspring. The experiments by BREEUWER and WERREN (1995) showed that hatching

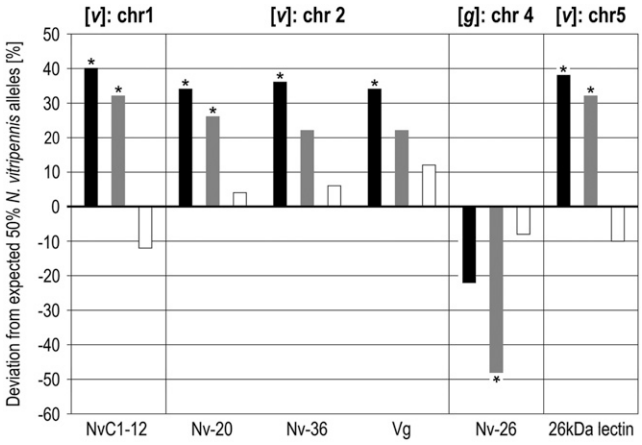

FIGURE 4.—Deviation of selected markers linked to predicted transmission ratio distorting loci in six populations of male F_2 hybrids of *N. giraulti* and *N. vitripennis* from the expected 50% *N. vitripennis* alleles. The genotype of the cytoplasm of a population is indicated by [*g*] = *N. giraulti* and [*v*] = *N. vitripennis*. Solid bars: deviation of the markers in the two populations ([*g*] and [*v*] cytoplasm, respectively) of adult wasps in the original experiment, which aimed to identify possible TRDL (asterisks indicate significance). Shaded bars: deviation of the same markers in two independent populations ([*g*] and [*v*] cytoplasm, respectively) of adult wasps studied in a second (confirmation) experiment (asterisks indicate significance). Open bars: deviation of markers in early (<16 hr old) hybrid embryos.

rates of male F_2 hybrids of *N. giraulti* and *N. vitripennis* do not significantly differ from those of the parental lineages. Rather, the increased mortality of F_2 hybrid males occurs during their larval development and during metamorphosis in the pupal stage. The asymmetry and direction of MTRD in the reciprocal crosses suggests genic incompatibilities between the nuclear genome and a maternally inherited genetic factor in the cytoplasm, most likely the mitochondrial genome. This interpretation is consistent with the Dobzhansky–Muller model for the evolution of genic incompatibilities (DOBZHANSKY 1934; MULLER 1939, 1940, 1942).

The Dobzhansky–Muller model for the evolution of genic incompatibilities predicts that hybrid incompatibilities are asymmetrical (MULLER 1942; ORR 1995; TURELLI and MOYLE 2007). While, for example, a *N. vitripennis* allele at locus A might be incompatible with a *N. giraulti* allele at locus B, the *N. vitripennis* allele of locus B cannot be incompatible with a *N. giraulti* allele of locus A (COYNE and ORR 2004). This is because the latter represents an intermediate step in the divergence of the two species. Assuming that the *N. giraulti* alleles at loci A and B represent the ancestral state, the derived state, *i.e.*, *N. vitripennis* alleles at loci A and B, would probably not have evolved had the intermediate state not been compatible. In the aforementioned example, one can easily replace "locus A" with one of the identified TRDL in *N. giraulti* × *N. vitripennis* F_2 hybrid males with *N. vitripennis* cytoplasm and "locus B" with a maternally inherited locus of the mitochondrial genome. The resulting pattern predicted by the Dobzhansky–Muller model for genic incompatibilities matches with our results.

If we assume that the observed MTRD is solely due to an increased mortality of individuals with the genotype in deficit, we can infer the mortality rate caused by a TRDL by calculating $m = 1 - \frac{1}{2}x$, where x is the estimated frequency of the parental allele occurring in excess. The survival rate s is then $1 - m$. Considering the Bayesian estimates for the MTRD of TRDL (Figure 3B), the mortality rate in F_2 hybrid males with *N. vitripennis* cytoplasm caused by the TRDL on chromosome 1 is with 95% pP of 15–39%, with the highest pP of 25–26%. The corresponding values for the TRDL on chromosomes 2, 4, and 5 are 9–36% (21–22%), 0–32% (14–15%), and 9–35% (21–22%), respectively (Figure 1). Since tests for significant deviation from the expected distribution of recombinant and nonrecombinant genotypes between markers linked to TRDL on chromosomes 1, 2, and 5 (TRDL1, TRDL2, TRDL5, respectively) in hybrids with *N. vitripennis* cytoplasm did not indicate TRDL interdependency, the overall mortality rate, m_0, of F_2 hybrid males with *N. vitripennis* cytoplasm caused by the three TRDL is $1 - [(1 - m_{\mathrm{TRDL1}}) \times (1 - m_{\mathrm{TRDL2}}) \times (1 - m_{\mathrm{TRDL5}})]$. Using the above-mentioned values, the three TRDL account with 95% pP for an overall mortality of 30–86%, with highest pP of 53–55%. In contrast, the TRDL on chromosome 4 in F_2 hybrid males with a *N. giraulti* cytoplasm explains 0–32% (95% pP; highest pP of 14–15%) mortality.

BREEUWER and WERREN (1995) inferred estimates of the survival rate of male F_2 hybrids of *N. giraulti* and *N. vitripennis*. According to their study, F_2 hybrid males with *N. vitripennis* cytoplasm have a survival rate of ~47% (±21 SD), while F_2 hybrid males with *N. giraulti* cytoplasm exhibit a survival rate of ~18% (±6 SD). A smaller study in our own laboratory revealed almost exactly the same values (results not shown). The mortality rate of F_2 hybrid males with *N. vitripennis* cytoplasm caused by the three identified TRDL was estimated to be between 30 and 86% (highest pP of 53–55%). Thus, the mortality caused by the three TRDL in F_2 hybrid males with *N. vitripennis* cytoplasm is sufficient to explain the observed survival rate of 47%. The mortality rate of the single identified TRDL in F_2 hybrid males with *N. giraulti* cytoplasm was estimated to be between 0 and 32%. This low rate stands in stark contrast to the low survival rate of the F_2 hybrid males with *N. giraulti* cytoplasm found by BREEUWER and WERREN (1995). The lower survival rate of male F_2 hybrids with *N. giraulti* cytoplasm in comparison to male F_2 hybrids from the reciprocal cross suggests that additional cytonuclear genic incompatibilities account for the increased mortality rate rather than pure nuclear–nuclear genic incompatibilities. There are several possible explanations of why we missed identifying these cytonuclear genic incompatibilities. First,

additional TRDL may exist but are in regions of the genome that are not covered by our genome map. The methods applied to estimate the genome coverage of the *N. girault*i × *N. vitripennis* linkage map in this study suggested that the linkage map spans 77% of the total genome and that 79 and 95% of the nuclear genome lay within 10 and 20 cM, respectively, of a mapped marker. However, these estimates had to be inferred under assumptions that the experimental data did not strictly fulfill (*e.g.*, uniform distribution of markers). The values therefore should be interpreted with caution. Second, the increased hybrid mortality may well be caused by TRDL that are in regions covered by our genome map, but the MTRD that they cause might be too small to be detected with the studied sample sizes. And third, the incompatibilities might be more complex and involve additional nuclear loci. Tests for a deviation from the expected distribution of recombinant and nonrecombinant genotypes between markers from different linkage groups in F_2 hybrids with a given cytoplasm did not reveal evidence for three-way interactions. However, three-way interactions, in which the two nuclear loci are on the same chromosome, were not tested. More complex interactions, involving more than two nuclear loci, are imaginable as well.

Gadau *et al.* (1999) reported that pure nuclear–nuclear genic incompatibilities are involved in *N. girault*i [*v*] × *N. vitripennis* F_2 hybrid breakdown as well. The authors compared the expected and the observed frequency of recombinant and nonrecombinant genotypes between markers from different chromosomes and found evidence for four possible nuclear–nuclear genic incompatibilities. Using the exact same approach, we failed to find comparable results in our study. Although an initial test of 120 F_2 hybrid males with *N. vitripennis* cytoplasm revealed notable deviations in five pairs of markers, we were not able to confirm them in a second hybrid population of the same size. The combined analysis of F_2 hybrid males from reciprocal crosses did not provide evidence for possible nuclear–nuclear genic incompatibilities either. In the latter, the sample size was equivalent to the one in the study by Gadau *et al.* (1999). One important difference between the two studies concerns the used strains. Gadau *et al.* (1999) used the *N. girault*i introgression strain R16A with *N. vitripennis* cytoplasm for their experiment while we used the cured and highly inbred *N. girault*i strain RV2X(U) with *N. girault*i cytoplasm. R16A was created by backcrossing males of the *N. girault*i strain RV2T for 16 generations to *N. vitripennis* females of the strain AsymC (Breeuwer and Werren 1995). Thus, although the nuclear genomes of R16A and RV2X(U) go back to RV2T and should theoretically have an identical nuclear genome, we assume that the R16A genome genetically changed due to intergenomic co-adaptation as a response to a heterospecific mitochondrial genome. A direct comparison of the results from these two studies is therefore difficult.

This study revealed that cytonuclear genic incompatibilities contribute significantly to hybrid breakdown in male F_2 hybrids of *N. girault*i and *N. vitripennis*. Which specific genes are incompatible remains to be investigated. Mishmar and Gershoni (2007) recently pointed out that pathways that are vital and undergo tight-and-fast co-evolutionary processes might be of particular significance for speciation. The mitochondrial oxidative phosphorylation (OXPHOS) pathway, the primary energy-generation system in aerobic metazoans, has characteristics that suggest its possible major role in speciation (Mishmar and Gershoni 2007). Four of the five OXPHOS enzyme complexes are composed of nuclear- and mitochondrial-encoded subunits. Disruption of the delicate interactions between the nuclear- and mitochondrial-encoded subunits introduced by hybridization is likely to affect mitochondrial ATP production capacity with far-reaching effects on the viability of the hybrids (Blier *et al.* 2001; Rand *et al.* 2004). Since the mitochondrial genome evolves much faster than the nuclear genome (Boore 1999; Lynch *et al.* 2006), incompatibilities are also expected to evolve rapidly between them (Mishmar and Gershoni 2007). Nuclear genes of the OXPHOS system are therefore promising candidates for being incompatible with a heterospecific mitochondrion. However, so far only a few studies have addressed the possible role of cytonuclear genic incompatibilities in hybrid breakdown (*e.g.*, Hutter 2002, 2007; Rawson and Burton 2002; Sackton *et al.* 2003; Burton *et al.* 2006; Ellison and Burton 2006; Harrison and Burton 2006; Willett 2006). The short generation time of Nasonia species, its haplodipolid sex determination, and availability of the genome sequence makes Nasonia a suitable model system for studying these interactions in further detail.

We are grateful to John H. Werren for sending us the Nasonia strains and the host puparia for culturing them. Benjamin Allen kindly cared for the Nasonia strains in the lab and conducted the control experiments of male F_2 hybrid hatching rates. Tom Dowling generously provided us with his NanoDrop spectrophotometer. We are further thankful to Claus Vogl for sharing his program ANITA with us and for his help with the Bayesian analyses. Joshua Gibson and two anonymous reviewers provided helpful comments on earlier drafts of the manuscript. O.N. acknowledges the Alexander von Humboldt Foundation for a Feodor Lynen postdoctoral stipend. This work was supported by a grant to J.G. from the National Institutes of Health (grant no. 1R21 RR024199-01).

LITERATURE CITED

Barbash, D. A., D. F. Siino, A. M. Tarone and J. Roote, 2003 A rapidly evolving MYB-related protein causes species isolation in *Drosophila*. Proc. Natl. Acad. Sci. USA **100:** 5302–5307.

Beukeboom, L. W., and C. Desplan, 2003 *Nasonia*. Curr. Biol. **13:** R860.

Blier, P. U., F. Dufresne and R. S. Burton, 2001 Natural selection and the evolution of mtDNA-encoded peptides: evidence for intergenomic co-adaptation. Trends Genet. **17:** 400–406.

Boore, J. L., 1999 Animal mitochondrial genomes. Nucleic Acids Res. **27:** 1767–1780.

Bordenstein, S. R., and J. H. Werren, 1999 Effects of A and B Wolbachia and host genotype on interspecies cytoplasmic incompatibility in Nasonia. Genetics **148:** 1833–1844.

Bordenstein, S. R., M. D. Drapeau and J. H. Werren, 2000 Intraspecific variation in sexual isolation in the jewel wasp *Nasonia*. Evolution **54:** 567–573.

Bordenstein, S. R., F. P. O'Hara and J. H. Werren, 2001 *Wolbachia*-induced bidirectional incompatibility precedes other hybrid incompatibilities in *Nasonia*. Nature **409:** 707–710.

Breeuwer, J. A. J., and J. H. Werren, 1990 Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. Nature **346:** 558–560.

Breeuwer, J. A. J., and J. H. Werren, 1993 Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis*. Genetics **135:** 565–574.

Breeuwer, J. A. J., and J. H. Werren, 1995 Hybrid breakdown between two haplodiploid species: the role of nuclear and cytoplasmic genes. Evolution **49:** 705–717.

Breeuwer, J. A. J., R. Stouthamer, S. M. Barns, D. A. Pelletier, W. G. Weisburg *et al.*, 1992 Phylogeny of cytoplasmic incompatibility micro-organisms in the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences. Insect Mol. Biol. **1:** 25–36.

Brideau, N. J., H. Flores, J. Wang, S. Maheshwari, X. Wang *et al.*, 2006 Two Dobzhansky-Muller genes interact to cause hybrid lethality in *Drosophila*. Science **314:** 1292–1295.

Burton, R. S., C. K. Ellison and J. S. Harrison, 2006 The sorry state of F_2 hybrids: consequences of rapid mitochondrial DNA evolution in allopatric populations. Am. Nat. **168:** S14–S24.

Cabot, E. L., A. W. Davis, N. A. Johnson and C.-I Wu, 1994 Genetics of reproductive isolation in the *Drosophila simulans* clade: complex epistasis underlying hybrid male sterility. Genetics **137:** 175–189.

Campbell, B. C., J. D. Steffen-Campbell and J. H. Werren, 1993 Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an rDNA internal transcribed spacer (ITS2). Insect Mol. Biol. **2:** 225–237.

Cazemajor, M., C. Landré and C. Montchamp-Moreau, 1997 The *sex-ratio* trait in *Drosophila simulans*: genetic analysis of distortion and suppression. Genetics **147:** 635–642.

Chakravarti, A., L. K. Lasher and J. E. Reefer, 1991 A maximum likelihood method for estimating genome length using genetic linkage data. Genetics **128:** 175–182.

Comai, L., K. Young, B. J. Till, S. H. Reynolds, E. A. Greene *et al.*, 2004 Efficient discovery of DNA polymorphisms in natural populations by Ecotilling. Plant J. **37:** 778–786.

Coyne, J. A., and H. A. Orr, 2004 *Speciation*. Sinauer Associates, Sunderland, MA.

Darling, D. C., and J. H. Werren, 1990 Biosystematics of *Nasonia* (Hymenoptera: Pteromalidae): two new species reared from birds' nests in North America. Ann. Entomol. Soc. Am. **83:** 352–370.

Delneri, D., I. Colson, S. Grammenoudi, I. N. Roberts, E. J. Louis *et al.*, 2003 Engineering evolution to study speciation in yeast. Nature **422:** 68–72.

Dobzhansky, T., 1934 Studies on hybrid sterility. I. Spermatogenesis in pure and hybrid *Drosophila pseudoobscura*. Z. Zellforsch. Mikrosk. Anat. **21:** 169–221.

Dobzhansky, T., 1975 Analysis of incipient reproductive isolation within a species of *Drosophila*. Proc. Natl. Acad. Sci. USA **72:** 3638–3641.

Drapeau, M. D., and J. H. Werren, 1999 Differences in mating behavior and sex ratio between three sibling species of *Nasonia*. Evol. Ecol. Res. **1:** 223–239.

Ellison, C. K., and R. S. Burton, 2006 Disruption of mitochondrial function in interpopulation hybrids of *Tigriopus californicus*. Evolution **60:** 1382–1391.

Fishmann, L., and J. H. Willis, 2005 A novel meiotic drive locus almost completely distorts segregation in Mimulus (Monkeyflower) hybrids. Genetics **169:** 347–353.

Fishmann, L., and J. H. Willis, 2006 A cytonuclear incompatibility causes anther sterility in *Mimulus* hybrids. Evolution **60:** 1372–1381.

Fishmann, L., A. J. Kelly, E. Morgan and J. H. Willis, 2001 A genetic map in the *Mimulus guttatus* species complex reveals transmission ratio distortion due to heterospecific interactions. Genetics **159:** 1701–1716.

Gadau, J., J. H. Werren and R. E. Page, 1999 Mapping of hybrid incompatibility loci in Nasonia. Genetics **153:** 1731–1741.

Gadau, J., J. H. Werren and R. E. Page, 2002 The genetic basis of the interspecific differences in wing size in Nasonia (Hymenoptera; Pteromalidae): major quantitative trait loci and epistasis. Genetics **161:** 673–684.

Gadau, J., O. Niehuis, A. Peire, J. H. Werren, E. Baudry *et al.*, 2008 The jewel wasp *Nasonia*, pp. 27–41 in *Genome Mapping and Genomics in Animals. IV. Insects*, edited by C. Kole and W. Hunter. Springer-Verlag, Berlin/Heidelberg, Germany, New York.

Ganetsky, B., 1977 On the components of segregation distortion in *Drosophila melanogaster*. Genetics **86:** 321–355.

Haldane, J. B. S., 1919 The combination of linkage values, and the calculation of distances between the loci of linked factors. J. Genet. **8:** 299–309.

Hall, M. C., and J. H. Willis, 2005 Transmission ratio distortion in intraspecific hybrids of *Mimulus guttatus*: implications for genomic divergence. Genetics **170:** 375–386.

Hall, T. A., 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:** 95–98.

Harrison, J. S., and R. S. Burton, 2006 Tracing hybrid incompatibilities to single amino acid substitutions. Mol. Biol. Evol. **23:** 559–564.

Harter, H. L., H. J. Khamis and R. E. Lamb, 1984 Modified Kolmogorov-Smirnov tests for goodness of fit. Comm. Stat. Simul. Comp. **13:** 293–323.

Hurst, L. D., 1993 A model for the mechanism of transmission ratio distortion and for *t*-associated hybrid sterility. Proc. R. Soc. Lond. Ser. B Biol. Sci. **253:** 83–91.

Hurst, G. D. D., and J. H. Werren, 2001 The role of selfish genetic elements in eukaryotic evolution. Nat. Rev. Genet. **2:** 597–606.

Hutter, P., 1997 Genetics of hybrid inviability in *Drosophila*. Adv. Genet. **36:** 157–185.

Hutter, P., 2002 X-linked small GTPase linked and OXPHOS genes are candidates for the genetic basis of hybrid incompatibility in *Drosophila*. Dev. Genes Evol. **212:** 504–512.

Hutter, P., 2007 Rapidly evolving *Rab* GTPase paralogs and reproductive isolation in *Drosophila*. Adv. Genet. **58:** 1–23.

Johnson, N. A., 2000 Gene interactions and the origin of species, pp. 197–212 in *Endless Forms: Species and Speciation*, edited by D. Howard and S. Berlocher. Oxford University Press, Oxford.

Khamis, H. J., 1990 The delta-corrected Kolmogorov-Smirnov test for goodness of fit. J. Stat. Plan. Inference **24:** 317–335.

Khamis, H. J., 1993 A comparative study of the delta-corrected Kolmogorov-Smirnov test. J. Appl. Stat. **20:** 401–421.

Korol, A. B., I. A. Preygel and Y. I. Nyutin, 1989 Quasi-linkage in tomato: formal analysis. Genetika **25:** 2225–2233.

Kosambi, D. D., 1944 The estimation of map distances from recombination values. Ann. Eugen. **12:** 172–175.

Lange, K., and M. Boehnke, 1982 How many polymorphic genes will it take to span the human genome? Am. J. Hum. Genet. **34:** 842–845.

Lavine, M., and M. J. Schervish, 1999 Bayes factors: what they are and what they are not. Am. Stat. **53:** 119–122.

Lewontin, R. C., 1974 *The Genetic Basis of Evolutionary Change*. Columbia University Press, New York.

Lilliefors, H. W., 1967 On the Kolmogorov-Smirnov test for normality with mean and variance unknown. J. Am. Stat. Assoc. **62:** 399–402.

Liu, B. H, 1998 *Statistical Genomics: Linkage, Mapping, and QTL Analysis*. CRC Press, Boca Raton, FL.

Lorieux, M., B. Goffinet, X. Perrier, D. González de León and C. Lanaud, 1995a Maximum likelihood models for mapping genetic markers showing segregation distortion. 1. Backcross populations. Theor. Appl. Genet. **90:** 73–80.

Lorieux, M., X. Perrier, B. Goffinet, C. Lanaud and D. González de León, 1995b Maximum likelihood models for mapping genetic markers showing segregation distortion. 2. F_2-populations. Theor. Appl. Genet. **90:** 81–89.

Lynch, M., B. Koskella and S. Schaack, 2006 Mutation pressure and the evolution of organelle genomic architecture. Science **311:** 1727–1730.

Lyttle, T. W., 1991 Segregation distorters. Annu. Rev. Genet. **25:** 511–557.

Malitschek, B., D. Fornzler and M. Schartl, 1995 Melanoma formation in *Xiphophorus*: a model system for the role of receptor tyrosine kinases in tumorigenesis. BioEssays **17:** 1017–1023.

Merçot, H., A. Atlan, M. Jacques and C. Montchamp-Moreau, 1995 Sex-ratio distortion in *Drosophila simulans*: co-occurrence of a meiotic drive and a suppressor of drive. J. Evol. Biol. **8:** 283–300.

Merrill, C., L. Bayraktaroglu, A. Kusano and B. Ganetzky, 1999 Truncated RanGAP encoded by the *Segregation distorter* locus of *Drosophila*. Science **283:** 1742–1745.

Mester, D. I., Y. I. Ronin, D. Minkov, E. Nevo and A. B. Korol, 2003a Constructing large-scale genetic maps using an evolutionary strategy algorithm. Genetics **165:** 2269–2282.

Mester, D. I., Y. I. Ronin, Y. Hu, J. Peng, E. Nevo *et al.*, 2003b Efficient multipoint mapping: making use of dominant repulsion-phase markers. Theor. Appl. Genet. **107:** 1102–1112.

Mester, D. I., Y. I. Ronin, E. Nevo and A. B. Korol, 2004 Fast and high precision algorithms for optimization in large-scale genomic problems. Comput. Biol. Chem. **28:** 281–290.

Mishmar, D., and M. Gershoni, 2007 Treating speciation processes as complex traits. Nat. Rev. Genet. **8:**.

Montchamp-Moreau, C., and D. Joly, 1997 Abnormal spermiogenesis is associated with the X-linked *sex-ratio* trait in *Drosophila simulans*. Heredity **79:** 24–30.

Moyle, L. C., and E. B. Graham, 2006 Genome-wide associations between hybrid sterility QTL and marker transmission ratio distortion. Mol. Biol. Evol. **23:** 973–980.

Muller, J. H., 1939 Reversibility in evolution considered from the standpoint of genetics. Biol. Rev. Camb. Philos. Soc. **14:** 261–280.

Muller, J. H., 1940 Bearing of the *Drosophila* work on systematics, pp. 185–268 in *The New Systematics*, edited by J. S. Huxley. Clarendon Press, Oxford.

Muller, J. H., 1942 Isolating mechanisms, evolution, and temperature. Biol. Symp. **6:** 71–125.

Niehuis, O., A. K. Judson, J. H. Werren, W. B. Hunter, P. M. Dang *et al.*, 2007 Species diagnostic single-nucleotide polymorphism and sequence-tagged site markers for the parasitic wasp genus *Nasonia* (Hymenoptera: Pteromalidae). J. Econ. Entomol. **100:** 1033–1036.

Orr, A., 1995 The population genetics of speciation: the evolution of hybrid incompatibilities. Genetics **139:** 1805–1813.

Orr, A., 2005 The genetic basis of reproductive isolation: insights from *Drosophila*. Proc. Natl. Acad. Sci. USA **102:** 6522–6526.

Orr, H. A., and S. Irving, 2001 Complex epistasis and the genetic basis of hybrid sterility in the *Drosophila pseudoobscura* Bogota-USA hybridization. Genetics **158:** 1089–1100.

Orr, H. A., and S. Irving, 2005 Segregation distortion in hybrids between the Bogota and USA subspecies of *Drosophila pseudoobscura*. Genetics **169:** 671–682.

Orr, A., J. P. Masly and D. C. Presgraves, 2004 Speciation genes. Curr. Opin. Genet. Dev. **14:** 675–679.

Pardo-Manuel de Villena, F., E. de la Casa Eperón, T. L. Briscoe and C. Sapienza, 2000 A genetic test to determine the origin of maternal transmission ratio distortion: meiotic drive at the mouse *Om* locus. Genetics **154:** 333–342.

Peng, J. H., A. B. Korol, T. Fahima, M. S. Röder, Y. I. Ronin *et al.*, 2000 Molecular genetic maps in wild emmer wheat, *Triticum dicoccoides*: genome-wide coverage, massive negative interference, and putative quasi-linkage. Genome Res. **10:** 1509–1531.

Pietsch, C., 2005 The genetics of species differences within the genus *Nasonia* Ashmead 1904 (Hymenoptera: Pteromalidae). D. Nat. Sci. Thesis, University of Würzburg, Würzburg, Germany.

Pietsch, C., K. Rütten and J. Gadau, 2004 Eleven microsatellite markers in *Nasonia*, Ashmead 1904 (Hymenoptera; Pteromalidae). Mol. Ecol. Notes **4:** 43–45.

Presgraves, D. C., L. Balagopalan, S. M. Abmayr and H. A. Orr, 2003 Adaptive evolution drives divergence of a hybrid inviability gene between two species of *Drosophila*. Nature **423:** 715–719.

Rand, D. M., R. A. Haney and A. J. Fry, 2004 Cytonuclear coevolution: the genomics of cooperation. Trends Ecol. Evol. **19:** 645–653.

Rawson, P. A., and R. S. Burton, 2002 Functional coadaptation between cytochrome *c* and cytochrome *c* oxidase within allopatric populations of a marine copepod. Proc. Natl. Acad. Sci. USA **99:** 12955–12958.

Rütten, K., C. Pietsch, K. Olek, M. Neusser, L. W. Beukeboom *et al.*, 2004 Chromosomal anchoring of linkage groups and identification of wing size QTL markers and FISH probes derived from microdissected chromosomes in *Nasonia* (Pteromalidae: Hymenoptera). Cytogenet. Genome Res. **105:** 126–133.

Sackton, T. B., R. A. Haney and D. M. Rand, 2003 Cytonuclear coadaptation in *Drosophila*: disruption of cytochrome c oxidase activity in backcross genotypes. Evolution **57:** 2315–2325.

Sawamura, K., Y. Tomimura, H. Sato, H. Yamada, M. Matsuda *et al.*, 2006 Establishing interspecific mosaic genome lines between *Drosophila ananassae* and *Drosophila pallidosa* by means of parthenogenesis. Genet. Res. **88:** 1–11.

Schartl, A., N. Dimitrijevic and M. Schartl, 1994 Evolutionary origin and molecular biology of the melanoma-inducing oncogene of *Xiphophorus*. Pigment Cell Res. **7:** 428–432.

Silver, L. M., 1993 The peculiar journey of a selfish chromosome: mouse *t* haplotypes and meiotic drive. Trends Genet. **9:** 250–254.

Skinner, S. W., and J. H. Werren, 1980 The genetics of sex determination in *Nasonia vitripennis* (Hymenoptera, Pteromalidae). Genetics **94:** s98.

Stebbins, G. L., 1950 *Variation and Evolution in Plants*. Columbia University Press, New York.

Tao, Y., and D. L. Hartl, 2003 Genetic dissection of hybrid incompatibilities between *Drosophila simulans* and *D. mauritiana*. III. Heterogeneous accumulation of hybrid incompatibilities, degree of dominance, and implications for Haldane's rule. Evolution **57:** 2580–2598.

Tao, Y., D. L. Hartl and C. C. Laurie, 2001 Sex-ratio segregation distortion associated with reproductive isolation in *Drosophila*. Proc. Natl. Acad. Sci. USA **98:** 13183–13188.

Ting, C.-T., S.-C. Tsaur, M.-L. Wu and C.-I Wu, 1998 A rapidly evolving homeobox at the site of a hybrid sterility gene. Science **282:** 1501–1504.

Turelli, M., and L. C. Moyle, 2007 Asymmetric postmating isolation: Darwin's corollary to Haldane's rule. Genetics **176:** 1059–1088.

Velthuis, B.-J., W. Yang, T. van Opijnen and J. H. Werren, 2005 Genetics of female mate discrimination of heterospecific males in *Nasonia* (Hymenoptera, Pteromalidae). Anim. Behav. **69:** 1107–1120.

Vogl, C., and S. Xu, 2000 Multipoint mapping of viability and segregation distorting loci using molecular markers. Genetics **155:** 1439–1447.

Werren, J. H., 1997 Biology of *Wolbachia*. Annu. Rev. Entomol. **42:** 587–609.

Werren, J. H., 1998 *Wolbachia* and speciation, pp. 245–260 in *Endless Forms: Species and Speciation*, edited by D. Howard and S. Berlocher. Oxford University Press, Oxford.

Weston, R. F., I. Qureshi and J. H. Werren, 1999 Genetics of a wing size difference between two *Nasonia* species. J. Evol. Biol. **12:** 586–595.

Whiting, A. R., 1967 The biology of the parasitic wasp *Mormoniella vitripennis* [= *Nasonia brevicornis*] (Walker). Q. Rev. Biol. **42:** 333–406.

Wilfert, L., J. Gadau and P. Schmid-Hempel, 2007 The genetic architecture of immune defense and reproduction in male *Bombus terrestris* bumblebees. Evolution **61:** 804–815.

Willett, C. S., 2006 Deleterious epistatic interactions between electron transport system protein-coding loci in the copepod *Tigriopus californicus*. Genetics **173:** 1465–1477.

Wittbrodt, J., D. Adam, B. Malitschek, W. Maueler, F. Raulf *et al.*, 1989 Novel putative receptor tyrosine kinase encoded by the melanoma-inducing *Tu* locus in *Xiphophorus*. Nature **341:** 415–421.

Wu, C.-I, T. W. Lyttle, M.-L. Wu and G.-F. Lin, 1988 Association between a satellite DNA-sequence and the responder of Segregation distorter in *Drosophila melanogaster*. Cell **54:** 179–189.

Yates, F., 1934 Contingency table involving small numbers and the χ^2 test. J. R. Stat. Soc. **1**(Suppl.)**:** 217–235.

Zar, J. H., 1999 *Biostatistical Analysis*, Ed. 4. Prentice-Hall, Englewood Cliffs, NJ.

Communicating editor: H. G. Spencer

APPENDIX

Characterization of nuclear and mitochondrial molecular markers in this investigation

Locus	Type	Primer sequences (5′ → 3′)	Amplicon length[a]	SNP position[a]	GenBank accession no.[a]	PCR temperature profile[c]	Reference
			Chromosome 1				
NvC1-20	LP	F: TGTAAAAGTAGTCCGCTTCG	~117/~125		–	I	Rütten *et al.* (2004)
		R: TATTTATATATGGAAAAAGAGG			–		
Cu/Zn SOD	SNP	F: AGTTCATGCCTTTGTGATGC	253	152 (A/G)	EF190557	III	Niehuis *et al.* (2007)
		R: CACCGACAAACAGATTCAGC			EF190556		
NvC1-21	P/A	F: GTAACAGTGAGATAAATGTG	−/~148		–	I	Rütten *et al.* (2004)
		R: TAGCAACGATAGTCCACG			–		
NvC1-6	LP	F: GGTTGCTTTTAAGTCTTTGC	~143/~141		–	I	Rütten *et al.* (2004)
		R: CTGGTCTTCTGCATAATGG			–		
NvC1-22	LP	F: GCAGAGTCGAGGCAAG	~214/~206		–	I	Rütten *et al.* (2004)
		R: TTACCGGAGTTCGTTAAC			–		
EF-1α F1	LP	F: GGTCATCGTCCTCAATCATC	219/214		EF190561	III	Niehuis *et al.* (2007)
		R: TTGTTTTACCTTTTCCCAACC			EF190560		
NvC1-12	LP	F: TCGCATTTTACATCTCTTTC	~91/~146		–	I	Rütten *et al.* (2004)
		R: GAGATAAACGAATCAAAAGAC			–		
NvC1-13	P/A	F: TAAAAGTATTAGACCTTTGGG	−/~115		–	iv	Rütten *et al.* (2004)
		R: AGTGGCTGAGCTTGGC			–		
			Chromosome 2				
Hex70b	SNP	F: GCAGCTTCTCAGGAGTCAGG	185	88 (G/T)	EF638397[b]	III	Gadau *et al.* (2008)
		R: AGGCATCATCTTCAGCAAGG		163 (C/T)	EF638396[b]		and this article
UQCRFS1	SNP	F: GCAACCTGAATGGTTGATTG	142	82 (A/T)	EF190571	III	Niehuis *et al.* (2007)
		R: TGGACCTTTCCTAATTCTTCC			EF190570		
Hsc70	SNP	F: AGGCCAGCATTGAAATCG	208	137 (T/C)	EF638394[b]	III	Gadau *et al.* (2008)
		R: TTCTGGATCTTGGGGATACG			EF638395[b]		and this article
Nv-20	LP	F: TGACGAAGTATCCGAGAAG	~87/~103		–	II	Rütten *et al.* (2004)
		R: TCGAAAAACGATATTGCTCG			–		
PTEN	LP	F: ACGGGTGTGATGGTATGTTG	232/235		EF638400[b]	III	Gadau *et al.* (2008)
		R: ACATACCTCCTTTGGGATGG			EF638401[b]		and this article
Nv-23	P/A	F: CAGCATACTCAAGCAAGC	−/~217		–	II	Rütten *et al.* (2004)
		R: GATACCTGAAGTTTGATGC			–		
Nv-36	LP	F: TCGATCCAGATGAAGAGG	~140/~168		–	I	Pietsch (2005)
		R: AGAGAATTAAGAGAAAGTCCG			–		
EF-1α F2	SNP	F: CCAGGACGTCTACAAGATTGG	249	106 (T/C)	EF638393[b]	III	Gadau *et al.* (2008)
		R: GGGTTGTTCTTGGAGTCACC		151 (A/T)	EF638392[b]		and this article
Vg	SNP	F: GGTCCTGACCAGAGAGAACG	191	25 (C/T)	EF638398[b]	III	Gadau *et al.* (2008)
		R: GCTCGAAGATTTCGAAGACG		75 (C/G)	EF638399[b]		and this article
TpnC	SNP	F: CTGAGGCTCTGGAGAAGGAG	257	121 (A/G)	EF190573	III	Niehuis *et al.* (2007)
		R: AAGTCGACGGTGCCAGAG			EF190572		
Nv-17	LP	F: AAGAATGTATCAAGTATGAGCC	~234/~215		–	I	Pietsch (2005)
		R: TCAGTTCTTGAAACGTTGC			–		
			Chromosome 3				
INSR	SNP	F: AAGATGTGGTCAAAGGCAAG	203	29 (T/G)	EF638386[b]	III	Gadau *et al.* (2008)
		R: ATTAAAGCCCCTGCAAAAAG		53 (T/C)	EF638387[b]		and this article
				124 (A/G)			
				163 (C/A)			
				178 (C/T)			
Hsp83	SNP	F: GCTCTTCCTCGTCGATTCC	215	178 (T/C)	EF190563	III	Niehuis *et al.* (2007)
		R: GAAGCACCTCGAGATCAACC			EF190562		
apoLp-III	LP	F: CCTTCCTGGAACTGCTTCTG	271/270		EF190555	III	Niehuis *et al.* (2007)
		R: TTCGTCAACAACGTCCAGAG			EF190554		
NvC3-18	P/A	F: GCCCAAATCATGCTTTCG	−/~105		–	II	Rütten *et al.* (2004)
		R: GTTGTTCTTAAATGTGTATTCC			–		
RPS2	SNP	F: TCAGCGTACTCGTTTCAAGG	256	141 (T/C)	EF190553	III	Niehuis *et al.* (2007)
		R: GCTTACCGATCTTGTTACCC			EF190552		

(*continued*)

APPENDIX

(Continued)

Locus	Type	Primer sequences (5′ → 3′)	Amplicon length[a]	SNP position[a]	GenBank accession no.[a]	PCR temperature profile[c]	Reference
SDR	LP	F: ACTGCTCTGCGCAATATGAC R: ATCAGAACAGCATCCGCTAC	321/307		EF638383[b] EF638382[b]	III	Gadau *et al.* (2008) and this article
TOR	LP	F: TCTTCCCATTCAACATCCAC R: TGACCATAAGCTGCGTCAC	174/175		EF638390 EF638391	III	Gadau *et al.* (2008) and this article
			Chromosome 4				
Nv-26	LP	F: TTCGCAGCTTTCCTTTGC R: AGCAGCTAGTATGAACCG	~117/~141		– –	II	Rütten *et al.* (2004)
Bb-77L07e	LP	F: GCCCGAAGATCTACATACGC R: CGATCCCAAATTACCTGCAC	227/259		EF638385[b] EF638384[b]	I	Gadau *et al.* (2008) and this article
Nv-24	LP	F: CCGAAATCCACATAGACC R: AGGAACTCATCAAGACGG	~98/~118		– –	II	Rütten *et al.* (2004)
PPAF-III	SNP	F: TGTCAAGCTCAAGGTCAACG R: TCCATACCACAGGGTGACG	237	49 (C/T)	EF190559 EF190558	III	Niehuis *et al.* (2007)
ACT	LP	F: TGCGAAACCAATTTCTTCTG R: AATGTGTATTAAAAGCACTTTCG	86/90		EF190569 EF190568	III	Niehuis *et al.* (2007)
NvC4-15	P/A	F: GCAGGGCTTTGTTATAGC R: CGACGAAACCGAAGTGG	−/~111		– –	I	Rütten *et al.* (2004)
COXVa	SNP	F: AATTCAATGTAAGGCTCGTTTC R: TGGATGAACTGGGTATCAGC	144	77 (C/G)	EF190567 EF190566	III	Niehuis *et al.* (2007)
			Chromosome 5				
26-kDa lectin	SNP	F: CGTATTTTTCAAGCCTCTCG R: TCGCAGATAAAAGCTCGTTC	153	35 (C/T)	EF190565 EF190564	III	Niehuis *et al.* (2007)
Nv-46	LP	F: TTACGTCAAGGTATAGCTGC R: AATAAGTGGCTGAAAGTTCC	~303/~332		– –	I	Pietsch (2005)
Nv-25	P/A	F: GTAAGTCTGCGGTAGCTG R: TTGACGGAGTAGTTCCAG	−/~238		– –	II	Rütten *et al.* (2004)
Ft	SNP	F: CAGCCTTCTATGCCAGTGTG R: CTGGTCGAAATGTAGAAAAAGC	156	64 (A/G) 84 (G/C) 87 (T/C)	EF638389[b] EF638388[b]	III	Gadau *et al.* (2008) and this article
Nv-27	LP	F: AATACTCGCTGTTCAATCG R: CGCTAGATCGGATTTCCG	~168/~192		– –	II	Rütten *et al.* (2004)
			Mitochondrion				
COI	SNP	F: CAACATTTATTTTGATTTTTTGG R: GCWACWACRTAATAKGTATCATG	385	70 (G/A) 190 (A/T)	EF638403[b] EF638402[b]	V	Modification from Gadau *et al.* (1999)

LP, length polymorphic marker; SNP, single nucleotide polymorphism marker; P/A, present/absent marker.

[a] The two sizes/alleles/accession numbers are for *N. giraulti* and *N. vitripennis*, respectively.

[b] Newly submitted sequences.

[c] I: 5 min at 95°, 10 cycles of 1 min at 94°, 1 min at 55° minus 1° each cycle, 1 min at 72°, followed by 25 cycles of 1 min at 94°, 1 min at 49°, 1 min at 72°; final 10 min at 72°. II: 5 min at 94°, 15 cycles of 1 min at 94°, 1 min at 60° minus 1° each cycle, 1 min at 72°, followed by 20 cycles of 1 min at 94°, 1 min at 50°, 1 min at 72°; final 10 min at 72°. III: 5 min at 95°, 30 cycles of 1 min at 95°, 1 min at 55°, 1 min at 72°; final 10 min at 72°. IV: 5 min at 95°, 30 cycles of 1 min at 95°, 1 min at 50°, 1 min at 72°; final 10 min at 72°. V: 4 min at 95°, 35 cycles of 1 min at 95°, 1 min at 45°, 1 min at 68°; final 4 min at 68°.