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THE INTERSPECIFIC ORIGIN OF B CHROMOSOMES: EXPERIMENTAL EVIDENCE

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Abstract.—A centric fragment was generated during the introgression of a chromosome region from *Nasonia girault*i into *N. vitripennis.* This neo B chromosome carries the *N. girault*i *or123*$^{+}$ gene for wild-type eye color. Using this phenotypic effect, the transmission of this chromosome was analyzed. The supernumerary chromosome showed less than Mendelian segregation rate in meiosis and some mitotic instability manifested as mosaic phenotype for eye color. However, transmission rate and mitotic stability increased over successive generations. The transmission rate through male gametogenesis was nearly 100%. These results support the interspecific hybridization model for B chromosome origin and reveal that problems in chromosome stability can persist for several generations after ''foreign chromosomes'' are introduced into a different species. We suggest that hybrid zones should be investigated as possible sites for neo-B chromosome generation.

Key words.—B chromosome evolution, interspecific hybrids, mitotic instability, *Nasonia vitripennis*.

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Supernumerary (or B) chromosomes are additional chromosomes that are found in a wide variety of plants and animals (Jones and Rees 1982, Beukeboom 1994). B chromosomes are nonvital (occurring in only some individuals within a species) and typically have accumulation mechanisms that increase their transmission prior, during, or following gametogenesis (e.g., Nur 1962).

The origin of B chromosomes is not clearly known. Two general hypotheses have been proposed: that B chromosomes arise from the standard A chromosomes (intraspecific origin), or that B's are derived from alien chromosomes introduced by interspecies mating (interspecific origin). There are only a few reported cases describing the emergence of new accessory chromosomes (for a review, see Camacho et al. 2000). For example, molecular evidence from the PSR chromosome in *Nasonia vitripennis* (a B chromosome causing all-male families) indicates that it has a hybrid origin (McAllister and Werren 1997).

A second important question concerns the fate of foreign chromosomes in interspecies hybrids. The ability of genetic material to move between species via hybridization and introgression depends in part upon the stability of foreign chromosomes in the genetic/cellular environment of the other species. Foreign chromosomes can be less stable and more prone to loss (Braverman et al. 1992) or fragmentation, due to such factors as meiotic and mitotic instability, sequence differences, inversions, transposition events, etc (e.g., Petrov et al. 1995). Therefore, fragmentation of foreign chromosomes is a possible mechanism by which hybridization results in neo-B chromosome generation (McVean 1995) as, for example, has been postulated for the PSR chromosome (McAllister and Werren 1997).

Nasonia is a genus of wasps that parasitize the pupae of various flies. *Nasonia vitripennis* and *N. girault*i are sibling species differing in some morphological and behavioral traits (Darling and Werren 1990). Formation of hybrids in these species is possible if endosymbiotic bacteria (*Wolbachia*) producing cytoplasmic incompatibility are previously eliminated (Breeuwer and Werren 1990). Partial F_2 hybrid breakdown occurs, resulting in mortality of 50–80% of F_2 (haploid) males (Breeuwer and Werren 1995; Gadau et al. 1999). However, lethal gene interactions are predominantly recessive, allowing introgression of chromosomal regions from one species to the other through hybrid females and surviving hybrid males.

Here we report the generation of a ''neo-B chromosome'' with phenotypic effects during the introgression of a foreign *N. girault*i chromosome into its sibling species *N. vitripennis.* This result is discussed in the light of B chromosome origin and evolution

MATERIAL AND METHODS

Cultures of *Nasonia* were maintained in the laboratory with constant light and temperature (25°C) on *Sarcophaga bullata* pupae. In these conditions generation time is approximately 14 days. The general biology of *Nasonia* is described by Whiting (1967).

Nasonia Strains

For this study we have used the following *Nasonia* lines, all of them were cured of *Wolbachia* by antibiotic treatment: (1) *or123R,* a *N. vitripennis* mutant line (eye color locus on linkage group IV) with orange-eyes instead of the wild-type dark brown eyes; (2) R16A, a *N. girault*i line produced by 16 generations of backcrossing *N. girault*i males to *N. vitripennis* Asymc females from an antibiotically cured strain (AsymC). This line has a virtually complete *girault*i genotype and *vitripennis* cytotype (e.g., mitochondria). The line is morphological and behaviorally identical to a *N. girault*i strain (Breeuwer and Werren 1995). The strain is wild-type at the *or123* locus (designated or_g^+/or_g^+).

Hybrid Introgression Regime

Crosses were conducted to introgress or_g^+, the wild-type dominant *N. girault*i allele at *or123* locus (plus flanking re-

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gions), into an otherwise vitripennis nuclear background. Initially, girault females (or_g^+/or_g^+) were crossed to *or123R* vitripennis males (or_v). In each generation, we selected heterozygous (or_g^+/or_v) hybrid females (wild-type dark brown eyes) and backcrossed these to *or123R* vitripennis males.

The experiment was originally designed to select for recessive hybrid lethal genes linked to or_g^+ while introgressing these into a vitripennis genetic background. Therefore, each generation or_g^+/or_v hybrid virgin females were provided with four *Sarcophaga* pupae for four days, and then the females were individually mated with *or123R* males and hosted with four new additional pupae. Due to haplodiploidy, virgin females produce only males that develop from unfertilized eggs. Recessive hybrid lethal genes linked to or_g^+ (i.e., *girault* hybrid lethals) result in lethality among some hybrid haploid males. To assay for hybrid lethals linked to or_g^+, families were scored for the ratio of ''or'' (or_v) to ''+'' (or_g^+) phenotypes among males, and daughters from families showing a distortion in the ''or'':''+'' ratio toward ''or'' were selected for the next generation. With this experimental approach, we introgressed a *girault* chromosome region carrying an eye color marker (or_g^+) and linked genes involved in hybrid breakdown, into a *vitripennis* genetic background. Theoretically, each generation of the introgression process halves the residual *girault* nuclear genome, assuming normal recombination and segregation.

Studies of the or_g^+ Low-Transmission Line

During the introgression procedure, an unusual line (INT-HD) showing a very low or_g^+ transmission ratio from heterozygous hybrid females (4.59% or_g^+ males) arose in the eighth backcross generation. This line was maintained as above.

Crosses were conducted to estimate the transmission of or_g^+ through males: 17 or_g^+ males were crossed to *or123R* females (or_v/or_v) and resulting female progeny were scored for eye color. Differential mortality between the male progeny of phenotypically or_v and or_g^+ virgin females was tested by individually placing 34 or_g^+ and 40 or_v females with two *Sarcophaga* pupae for two days. Then, we transferred each female to a *Sarcophaga* pupa in a foam plug, which exposed only the anterior tip of the pupa to the female, for five h. These parasitized *Sarcophaga* pupae were divided into two groups. The pupae from one group were cracked out and the number of eggs counted. The remaining pupae were left alone and the number of adult progeny was counted. Survival probability from egg to adult was estimated by dividing adult numbers by egg numbers. Differences in survivability were tested by *t*-tests, assuming the variance in survivability was equal to $var\ (Y)/\mu_x^2 + var\ (X)\mu_y^2/\mu_x^3$, where X stands for the number of eggs and Y for the number of adults (Breeuwer and Werren 1995).

The karyotype of INT-HD line males was examined cytologically by dissecting out testis of 10-day-old male pupae in *Drosophila* Ringers solution. Testes were maintained half an hour in a solution containing 0.8% KCl and 0.2% colchicine, and then fixed for 10 min. in Carnoy's fixative. After removing the fixative, testes were stained in acetic orcein and visually examined under 1000× microscope.

RESULTS

During the original introgression experiment of or_g^+ into a vitripennis nuclear background, lines were selected that showed a distorted recovery of or_v relative to or_g^+ among hybrid males. Results of this general experiment will be reported elsewhere (F. Perfectti, J. Gadau and J. H. Werren, unpubl. ms.). However, in the eight backcross generation (BC_8), we found one family that showed an extreme bias toward ''or'' versus ''+'' phenotypes among both sons (haploid) and daughters (diploid). The mother produced 187 ''or'' males and only nine ''+'' males, that is, the transmission of the or_g^+ allele was only 4.59%, instead of the expected 50% for a heterozygous female ($\chi^2 = 161.65$, $P < 0.001$). When this female was mated with an *or123R* male, her female progeny also showed a distorted segregation, 107 phenotypically ''or'' and 13 phenotypically ''+'' females (or_g^+ allele transmission = 10.83%, $\chi^2 = 73.63$, $P < 0.001$). The line was therefore studied to determine the cause of this distortion.

Several possibilities existed to explain the results. First (our favored hypothesis at the time), the extreme distortion could be due to a recessive hybrid lethal tightly linked to the *or123* locus. Hybrid lethal genes do not cause lethality in their own species background, and therefore are generally believed to cause lethality in interaction with other loci, usually with alleles from the other species (Dobzhansky 1936; Muller 1939; but see Gadau et al. 1999).

A second hypothesis is that introgression of or_g^+ into *vitripennis* could have released a meiotic drive locus linked to the *or123* locus. In this scenario, or_g^+/or_v heterozygous females show a distorted ratio because either or_v is preferentially transmitted to the functional pole during meiosis in eggs (chromosomal meiotic drive) or or_g^+ gametes are disabled (genic meiotic drive).

Third, the distortion could be due to production of a chromosomal fragment containing the centromere and including the or_g^+ locus. Centric fragments are known to occur in *Nasonia,* and show reduced transmission (5–30%) through meiosis, possibly due to inefficient segregation (Ryan et al. 1987; Beukeboom and Werren 1993; Perrot-Minnot and Werren 2001). Consistent with the fragment hypothesis, we found seven mosaic individuals for eye color (0.08%, $n = 8310$) in the INT-HD line. These individuals (five males and two females) showed eyes with both orange and the wild-type brown color sectors. Somatic mosaicism is a phenomenon observed with neo-B chromosomes, due to mitotic instability (Ryan et al. 1985; Beukeboom and Werren 1993).

Cytological Studies

To investigate the possible presence of centromeric fragment (fr^+)carrying the or_g^+ allele, we cytologically analyzed spermatocytes of phenotypically ''*or*'' and ''+'' males from the INT-HD line. The normal haploid complement of *Nasonia* is five chromosomes. In spermatocytes of ''+'' males, we consistently found a small centromeric fragment, in addition to the normal complement (Fig. 1). In ''*or*'' phenotype males from the same families, however, this fragment was not present. We can therefore conclude that a new centromeric fragment was generated in this line during introgression and that this microchromosome carried a region with the girault

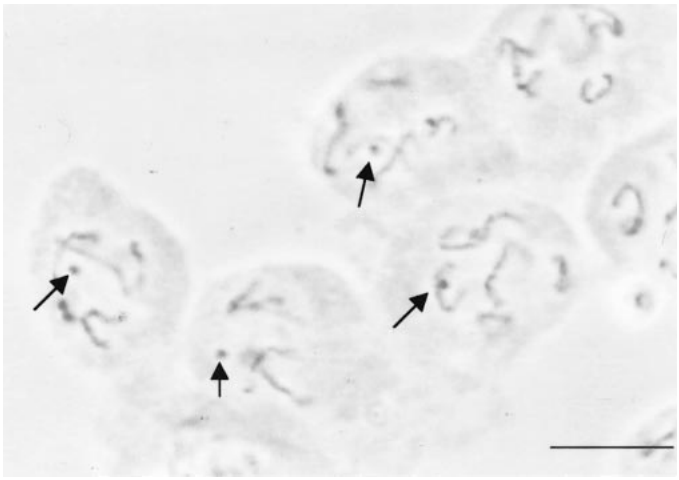

FIG. 1. Testis cells showing the neo-B chromosome (arrows). Bar = 10 μm.

or_g^+ gene conferring wild-type eye color as the main phenotypical effect. Centric fragements containing functional eye-color loci have also been observed for the R-complex of *Nasonia*, which occurs on Linkage Group I (Ryan et al. 1985). However, in this case we found a centric fragment bearing an eye-color locus of *or123*, which occurs on Linkage Group IV of *Nasonia.*

Transmission of the Fragment through Females

The neo-B carrying INT-HD line was continued for five more generations. In each generation phenotypically ''+'' females (bearing the or_g^+ fragment) were hosted as virgin and, after four days, mated with an *or123R vitripennis* male. Then they were hosted with four additional new *Sarcophaga* hosts, as previously described. Table 1 shows the segregational data in these five generations. The average transmission rate of or_g^+ fragment from females was 19.30% (95% confidence interval: 18.36–20.27%) to sons and 20.08% (95% confidence interval: 18.22–22.04%) to daughters (values not significantly different: $\chi^2 = 0.535$, $P = 0.465$).

An increase in the transmission rate of the or_g^+ fragment from females to their progeny was observed during the next five generations both to sons and daughters, but this was only significant among sons (Pearson correlation $r = 0.837$, $t_4 = 3.059$, $P = 0.038$).

Transmission through Males

Transmission of the fragment through males was measured by crossing ''+'' males (genotypically $or_v fr^+$) with *or123* females (or_v/or_v). Recall that males are haploid and have mitotic gametogenesis. Therefore, this is a measure of mitotic stability. We obtained female progeny in 14 out of 17 crosses (mean family size = 64.43 ± 3.03 females, two *Sarcophaga* hosts). The or_g^+ fragment transmission was 95.57% (95% confidence interval: 94.02–96.73%; range: 84.85–100%). This value indicates that the transmission through males is greater than the transmission through females. This is most likely due to higher transmission through the mitotic gametogenesis of haploid males, versus meiotic gametogenesis of females, and has been observed in other B chromosomes in *Nasonia* (Beukeboom et al. 1992; Perrot-Minnot and Werren 2001).

Fecundity and Survival in INT-HD Line

Fecundity was similar for both phenotypically ''or'' females (i.e., genotypically or_v/or_v) and phenotypically ''+'' females (i.e., genotypically $or_v/or_v fr^+$). Females were permitted to oviposit during five hours. After this time, the or_v/or_v females oviposited a mean of 38.50 ± 3.50 eggs and $or_v/or_v fr^+$ females oviposited a mean of 40.21 ± 3.61 eggs. These values were not significantly different ($t_{30} = 0.337$, $P = 0.739$).

Survival probability from egg to adult was estimated for the male progeny of both or_v/or_v females and $or_v/or_v fr^+$ females. The survival probability of the male progeny was 0.76 ± 0.31 for or_v/or_v females and 0.72 ± 0.31 for $or_v/or_v fr^+$ females. These values were not significantly different ($t_{30} = 0.954$, $P = 0.348$).

DISCUSSION

The results described here have implications for two important issues, (1) origin of B chromosomes, and (2) stability

TABLE 1. Transmission of the or_g^+ chromosome in *N. girault* × *N. vitripennis* hybrid line INT-HD. BC followed by a numeral indicates the backcross generation. Number of families (*n*) is indicated, as is the percent transmission and 95% confidence limits (in parentheses).

	Male progeny				Female progeny			
Generation	*n*	or_v ♂	or_g^+ ♂	Transmission rate (%)	*n*	or_v ♀	or_g^+ ♀	Transmission rate (%)
0 (BC_8)	1	187	9	4.59 (2.43–8.50)	1	107	13	10.83 (6.44–17.66)
1 (BC_9)	7	1095	201	15.51 (13.64–17.58)	4	272	78	22.29 (18.24–26.93)
2 (BC_{10})	10	1659	402	19.51 (17.85–21.27)	5	221	70	24.05 (19.50–29.28)
3 (BC_{11})	10	1306	339	20.61 (18.72–22.63)	8	455	122	21.14 (18.01–24.66)
4 (BC_{12})	3	406	90	18.15 (15.00–21.78)	2	211	17	7.46 (4.71–11.62)
5 (BC_{13})	9	671	232	25.69 (22.95–28.64)	1	103	44	29.93 (23.12–37.77)

of chromosomes in hybrid genomes. B chromosomes are widespread and common in plants and animals. However, the major mechanisms causing B chromosome generation and evolution are still unclear. Battaglia (1964) first proposed that B chromosomes can arise as by-products of hybridization at intra- or interspecific levels. New evidence has supported this hypothesis (e.g., Sapre and Deshpande 1987; Schartl et al. 1995). In *Nasonia*, the only described B chromosome, PSR, apparently came from a hybridization event involving the closely related genus *Trichomalopsis* (McAllister and Werren 1997).

Here we show a newly generated supernumerary chromosome carrying a phenotypic marker in an introgression line between *Nasonia vitripennis* and *N. giraulti*. We can refer to this minichromosome as a ''neo-B chromosome.'' This neo-B chromosome is most likely of *N. giraulti* origin, because it carries the giraulti *or123*$^{+}$ gene for wild type eye color. The neo-B chromosome arose in the eighth backcross generation.

Only centric fragments with a centromeric region can be maintained and stably inherited. Centric fragments have been generated in *Nasonia* using cytoplasmic incompatibility (Ryan et al. 1987; Breeuwer and Werren 1993), X irradiation (Beukeboom and Werren 1993) and chemical mutagenesis (Perrot-Minnot and Werren 2001). In each case, centric fragments show a low transmission through females (6–24%) and a relatively high transmission through males (11–86% for centric fragments generated by chemical mutagenesis and 48–100% for irradiated PSR chromosomes). The neo-B chromosome described here was transmitted by nearly 100% of sperm of carrier males, suggesting that this neo-B chromosome behaved as a normal (A) chromosome in mitosis. However, its segregation through females was lower than Mendelian. The likely explanation for this is the difference in gametogenesis in the two sexes. Males are haploid and undergo a mitotic gametogenesis. Therefore, there is no reductional division. Females are diploid and undergo meiotic gametogenesis. Absence of a ready pairing partner for the neo-B chromosome, preferential inclusion into polar bodies, or failing to reach the ovum probably explain its low transmission through meiosis. Centric fragments may be more prone to lagging at the divisional plane and therefore fail to be included into the viable daughter pronuclei. Because a systematic cytological examination of female meiosis has not yet been conducted, we do not know to what extent centric fragments form trivalents with homologs or remain unpaired. However, Perrot-Minnot and Werren (2001) found that meiotic transmission of a neo-B chromosome was different when paired with homologous chromosomes bearing different mutations (and possible rearrangements affecting pairing efficiency). This result suggests that trivalents with homologous chromosomes are formed.

It is interesting that the rate of meiotic transmission increased during successive generations, although this increase could be a by-product of the line maintenance procedure or a line effect. The neo-B chromosome showed, initially, some mitotic instability as well, as evidenced by incomplete transmission through males and occurrence of eye color mosaicism in some individuals. However, after five generations of maintenance, nearly 100% mitotic stability was found. It is possible that both the increased mitotic and meiotic stability is due to the acquisition of telomeres by the fragment, although this was not explicitly investigated. However, without an accumulation mechanism (e.g., chromosomal drive), pairing with an homologue chromosome, or a fitness advantage, these extra chromosomes would be lost in natural populations due to less than 50% transmission through females (Öestergren 1945; Nur 1977). Another possibility for its subsistence is the incorporation of part of the chromosome fragment into another chromosome by a fusion event (e.g., Henriques-Gil et al. 1983).

Few B chromosomes are known to affect external phenotype (for a review, see Camacho et al. 2000). Exceptions include the maize B that induces striped leaves (Staub 1987), and the minichromosomes in *Poecilia formosa* producing pigmentation mosaicism (Schartl et al. 1995). Neo-B chromosomes may usually have coding regions, but without recombination they are expected to be pruned to mutational inactivation and heterochromatinization (McVean 1995), unless under strong positive selection. The phenomenon of polysomy in grasshoppers (e.g., Peters 1981) shows that de novo extra chromosomes produced each generation are promptly heterocromatinizated and not inherited. However, the neo-B chromosome of *Nasonia* seemed to show gene activity ever after five generations, which indicates that some condition for heterochromatinization and inactivation were not met. The observation that this centric fragment also contains the *or123* locus suggests that this locus is located relatively near the centromere, a finding consistent with other molecular genetic studies (Gadau et al. 1999).

Here we have found that centric fragments are generated when a chromosome from one species is introgressed into the other species. This was detected simply because we were screening for unusual transmission ratios of a marker during introgression. Interestingly, the chromosome breakage occurred after seven generations of backcrossing the foreign *N. giraulti* chromosome into an *N. vitripennis* background. We do not know whether this is a common occurrence during hybrizidation, but if it is, then the effect could reduce the movement of genes between species, and provide a source for B chromosome generation. Instability of chromosomes in a foreign genetic environment could be caused by transposon release (Petrov et al. 1995), pairing disruption due to sequence differences (e.g., tandemly arranged repetitive DNA) or other mechanisms. It is currently unknown how commonly interspecies hybridizations and movement of chromosomes into foreign genetic backgrounds leads to neo-B generation. However, this could be a common phenomenon in hybrid zones and is worthy of further investigation.

In conclusion, a centric fragment was generated during introgression of a chromosome region from one *Nasonia* species into the other. Our results support the interspecific hybridization model for B chromosome origin and indicate that problems in chromosome stability can persist for many generations when foreign chromosomes are introduced into a different species.

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