

Microorganisms associated with chromosome destruction and reproductive isolation between two insect species

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MICROORGANISMS have been implicated in causing cytoplasmic incompatibility in a variety of insect species, including mosquitoes, fruitflies, beetles and wasps¹⁻⁴. The effect is typically unidirectional: incompatible crosses produce no progeny¹⁻⁴ or sterile males⁵, whereas the reciprocal crosses produce normal progeny. The parasitic wasp *Nasonia vitripennis* is one of the few species in which the cytogenetic mechanism of incompatibility is known. In this species the paternal chromosome set forms a tangled mass in a fertilized egg and is eventually lost⁶. Here we report that cytoplasmic microorganisms are associated with complete bidirectional incompatibility between *N. vitripennis* and a closely related sympatric species, *N. giraulti*. Microorganisms can be seen in the eggs of both species. Hybrid offspring are normally not produced in crosses between the two species, but do occur after elimination of the microorganisms by antibiotic treatment. A cytogenetic and genetic study shows that bidirectional interspecific incompatibility is due to improper condensation of the paternal chromosomes. Microorganism-mediated reproductive isolation is of interest because it could provide a rapid mode of speciation^{8,9}. The mechanism of incompatibility in *Nasonia* is also of interest as a potential tool for studying chromosome imprinting and chromosome condensation.

Nasonia vitripennis is a small wasp which lays its eggs in the pupae of various fly species^{2,22}. As in other bees and wasps, *N. vitripennis* has haplodiploid sex determination. Males typically develop from unfertilized (haploid) eggs, whereas females develop from fertilized (diploid) eggs. Three species of *Nasonia* have been described that can be distinguished on morphological, behavioural and genetic criteria²²: *N. longicornis* occurs in western North America, *N. giraulti* occurs in eastern North America and *N. vitripennis* is cosmopolitan. *N. vitripennis* is found micro-sympatrically with each of its sibling species. Indeed, different species have been observed to emerge from the same patch of host pupae and even from the same host puparia²². Therefore, the potential for interspecific hybridization is real.

A study was originally undertaken to determine whether crosses between *N. vitripennis* (Nv) and *N. giraulti* (Ng) produced fertile hybrid offspring. Strains of each species differ in the degree of interspecific premating isolation (J.H.W. and M. Riley, unpublished results). Therefore we used an Ng strain (NgVa002) for initial studies of hybridization between Nv and Ng that readily mates with Nv, and a standard Nv strain (Lab 11). Although matings between the two species readily occurred, interspecific matings yielded only all-male progeny. Because only females are hybrids in haplodiploid species, no hybrid offspring were produced.

All-male progeny could have arisen from these crosses for at least five reasons: (1) absence of copulation (virgin females produce all-male offspring); (2) inadequate insemination or failure of the sperm to reach the spermatheca; (3) failure of sperm to fertilize eggs; (4) post-fertilization incompatibility, resulting in loss of paternal chromosomes, or (5) hybrid (female) mortality. Possibilities (1) and (2) are eliminated because copulations were observed in interspecific matings, and dissection of females after mating revealed the presence of sperm in the spermatheca. Possibility (5) is eliminated because offspring numbers were not significantly reduced in interspecific matings

TABLE 1 Per cent hybrids (females)* and offspring number± for crosses using Symbiont and Asymbiont strains

Male parent	Female parent			
	Symbiont		Asymbiont	
	Nv	Ng	Nv	Ng
Symbiont				
Nv	66±11%	Of0%	Of0%	Of0%
Ng	Of0%	84±14%	0±0%	0±0%
Asymbiont				
Nv	69±8%	76±8%	75±4%	80±9%
Ng	20±22%	82±25%	19±24%	83±7%
Symbiont				
Nv	45.9±8.3 (27)	34.2±8.0 (30)	54.8±8.8 (10)	33.6±7.7 (12)
Ng	47.9±7.8 (28)	34.9±8.6 (24)	41.6±11.4 (24)	25.8±10.2 (17)
Asymbiont				
Nv	39.4±7.2 (5)	25.2±8.1 (28)	40.0±10.5 (11)	29.1±6.5 (10)
Ng	42.5±8.3 (15)	39.1±7.2 (12)	39.9±10.6 (20)	37.1±9.2 (13)

* The per cent of hybrids (females) (± s.d.) are given in the upper part of the table for crosses using Symbiont (S) and Asymbiont (A) strains.

† Offspring number (± s.d.) are shown in the lower part of the table for crosses using Symbiont and Asymbiont strains.

Sample sizes (shown in parentheses in the lower part of the table) are number of replicates in which four females are given four hosts. Recall that in haplodiploid organisms, only females are genetic hybrids, whereas the male is uniparentally derived. Offspring numbers are not reduced in interspecific crosses: NgS d xNvS Y versus NvS d xNvS Y ($P>0.60$) and NvS d xNgS Y versus NgS d xNgS Y ($P>0.60$; Mann-Whitney U-test). Hybrids are not produced in S d XS Y crosses, but are produced in A d XS Y crosses and A d x A Y crosses. A proportion of hybrids are produced in NgA d xNvA and NvS Y crosses that is significantly lower than in reciprocal crosses ($P<0.001$; Mann-Whitney U-test). This may be due to a lower mating propensity of Nv Y for Ng d, rather than to differences in the degree of compatibility.

An Asymbiont strain of Ng was produced by feeding (NgVa002) adult females a solution of 1 mg ml⁻¹ tetracycline in 10% sucrose and then allowing the females to lay eggs for 48 h. The procedure was repeated with the progeny for three more generations, at which time no bacteria could be detected within the eggs of this Ng Asymbiont line. The experiments were conducted 2 months (4 generations) after curing the Ng strain. An Nv asymbiont strain was used that had been cured of the Symbiont two years previously. Examination of eggs showed that the symbionts were still absent in both strains. Virgins were allowed to mate for 24 h (2 males x 4 females). After mating, females were placed on four hosts (*Sarcophaga bullata* pupa) and allowed to lay eggs. The subsequent number of offspring and sex ratio were scored.

Saul and colleagues¹⁻¹⁷ previously discovered unidirectional cytoplasmic incompatibility between laboratory strains of *N. vitripennis*, and demonstrated cytogenetically that the paternal chromosomes fail to condense properly and are eventually lost in incompatible crosses. Because of haplodiploid sex determination, the incompatible crosses produce all-male progeny. They further established that incompatibility was caused by an agent that could be eliminated by tetracycline treatments and reinoculated into previously cured lines¹⁷.

We therefore undertook a series of experiments to determine whether the absence of hybrid progeny in crosses between these species is caused by cytoplasmic incompatibility. First, we conducted a cytogenetic examination to follow the developmental events after egg fertilization in interspecific crosses (for procedures, see Fig. 1 legend and ref. 23). In both intra- and interspecific fertilized eggs, spermatozoa were easily detected. But by the first mitotic (cleavage) division, a dramatic difference was found between fertilized eggs from intraspecific and interspecific crosses. A normal diploid complement was typically present in eggs from intraspecific matings, but in fertilized eggs

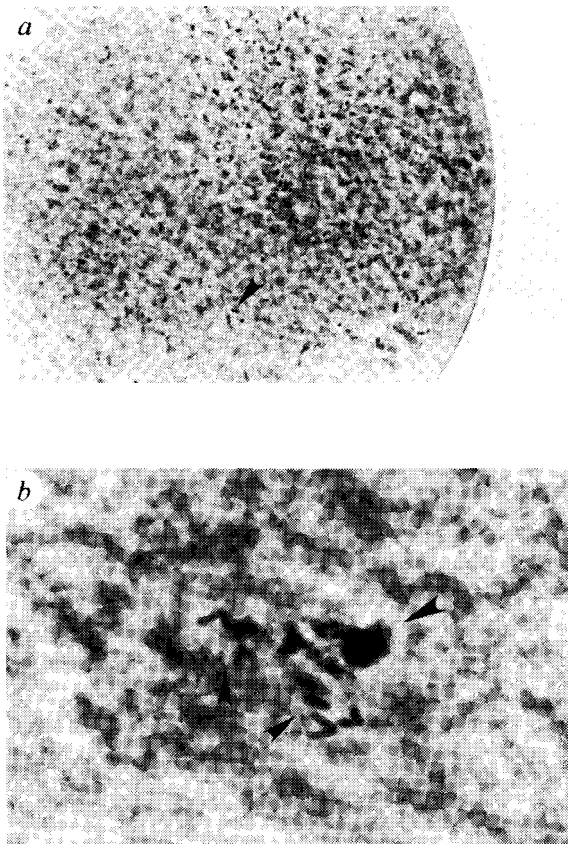


FIG. 1 *a*, Cytoplasmic microorganisms within an egg of *N. giraulti*. Bacteria are present throughout the posterior pole of the egg. Arrow indicates a dividing bacterium. These bacteria are absent in tetracyclin-treated strains. Although the species of microorganism is unknown, cytoplasmic incompatibility in several other insects is caused by rickettsia5-11. By counting bacteria in thirteen 0.01 mm x 0.01 mm columns through the egg and extrapolating to the total region containing bacteria within the egg, numbers of bacteria per standard Nv (Lab II) eggs are crudely estimated to be 1,903 \pm 476 s.d. (n=6). For a standard Ng (Va002), strain counts are 2,143f 1,017 s.d. (n=15). *b*, The appearance of improperly condensed chromosomes in anaphase of the first mitotic division of an incompatible cross between *N. giraulti* female and *N. vitripennis* male. The large arrow indicates the improperly condensed chromosome set. Two smaller arrows indicate dividing chromosomes. Based upon the genotype of male offspring resulting from such crosses, it can be concluded that it is the paternal chromosome set that fails to condense properly and is eventually lost. These cytogenetic events are similar to those described for unidirectionally incompatible crosses between *N. vitripennis* strains¹⁵⁻¹⁷.

METHODS. *a*, Eggs, collected between 0 and 70 min after oviposition by the wasp, were placed in Carnoy's fixative (acetic acid: chloroform: 95% ethanol in proportions 1:2.5:3). One to three days after fixation, eggs were transferred to a drop of 70% ethanol and mechanically stripped of their chorion and then stained in 2% lacmoid. *b*, Conditions as in *a*, except 2% aceto-lactic orcein was used for staining.

from interspecific matings, one set of chromosomes formed a diffuse tangled chromatin mass (Fig. 1). Use of an eye-colour mutant confirmed that the paternal chromosomes were destroyed in interspecific crosses. Bacteria can be seen in the eggs of normal (Symbiont) strains of both species, but are absent in antibiotic-cured (Asymbiont) strains (see Fig. 1 legend for method). The coccoid bacteria are clustered at the posterior pole of the egg (Fig. 1). This is the region that typically develops into germ-line tissues in insects.

To determine whether the symbiont microorganisms have a role in reproductive isolation between the sibling species, intra- and interspecific crosses were performed using Symbiont and Asymbiont strains. For each species, the Symbiont strain used in the experiments is the same one from which the Asymbiont strain was derived, so they are genetically identical. Results (Table 1) show that within each species, Symbiont males are incompatible with Asymbiont females, whereas the reciprocal cross is compatible. This is the same unidirectional cytoplasmic incompatibility pattern found within many other insect species¹⁵⁻¹⁷.

Interspecific crosses yield a dramatic result. Whereas crosses between the Symbiont (standard) strains of the two species result in all-male offspring, crosses using Asymbiont males of one species with either Symbiont or Asymbiont females from the other species results in production of hybrid (female) offspring. Thus, we conclude that elimination of the microorganism allows the two species to produce viable hybrids. Hybrid (female) offspring from both interspecific crosses are fertile, although there is increased mortality among their offspring (data not shown). Therefore, the species have diverged sufficiently genetically to produce partial hybrid breakdown.

It has been suggested that Symbionts that cause cytoplasmic incompatibility play a part in the process of speciation by preventing gene flow between populations^{18,19}. Caspari and Watson²⁴, however, showed with a population model that unidirectional incompatibility cannot easily result in reproduc-

tive isolation because of the rapid spread of the symbiont cytotype. By contrast, symbionts causing bidirectional incompatibility can lead to reproductive isolation between populations and ultimately to speciation.

The symbiont microorganisms may have facilitated speciation in *Nasonia* by causing reproductive isolation. Whether they still actively maintain reproductive isolation between these species depends on the frequency of interspecific matings. The two species are now sympatric over much of their range. Strains of the two species differ in degree of premating isolation (under laboratory conditions), suggesting that this character is actively evolving, perhaps in response to incompatibility. But we do not know whether interspecific mating is frequent in nature.

The bidirectional incompatibility between *Nasonia* species described here causes complete reproductive isolation that can be removed by eliminating an associated microorganism. Other examples of bidirectional incompatibility occur between strains of *Culex pipiens*^{25,26}, and between semi-species in *Drosophila paulistorum*^{12,14}. The number of documented cases of cytoplasmic incompatibility within insect species is rapidly growing as new species are examined¹⁵. On the basis of our findings, we suggest that cytoplasmic incompatibility between closely related arthropod species may be more widespread and significant than has previously been believed²⁰.

The biochemical mechanisms by which a symbiont can cause improper condensation of its eukaryotic host's chromosomes is an unknown and intriguing aspect of this system. Chromosomal imprinting is likely to be involved. *N. vitripennis* harbours (in some populations) a supernumerary chromosome^{23,27} I_n that cause destruction of the paternal chromosomes¹⁵ that cause the chromosomes appear to hypercondense, whereas cytoplasmic incompatibility results in undercondensation. These non-mendelian elements could prove useful in studying molecular mechanisms of chromosome imprinting and chromosome condensation, as well as in elucidating modes of speciation. E

Received 4 December 1989; accepted 25 May 1990.

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ACKNOWLEDGEMENTS. We thank L. Beukeboom, D. Eickbush, D. Ferris, J. Jaenike, D. Miller, U. Nur, M. Riley, R. Stouthamer and M. Vig for comments and assistance. Support was provided by the NSF, the NIH and Donders Fonds.