Copyright © 1997 by the Genetics Society of America

# Reproductive Isolation and Morphogenetic Evolution in Drosophila Analyzed by Breakage of Ethological Barriers

**Lucas Sánchez* and Pedro Santamaria†**

*Centro de Investigaciones Biológicas, Velázquez 144, 28006 Madrid, Spain and †Centre de Génétique Moleculaire du CNRS, 91198 Gif-sur-Yvette, Cedex, France

Manuscript received January 9, 1997
Accepted for publication June 13, 1997

ABSTRACT

This article reports the breaking of ethological barriers through the constitution of soma-germ line chimeras between species of the melanogaster subgroup of Drosophila, which are ethologically isolated. Female *Drosophila yakuba* and *D. teissieri* germ cells in a *D. melanogaster* ovary produced functional oocytes that, when fertilized by *D. melanogaster* sperm, gave rise to sterile *yakuba-melanogaster* and *teissieri-melanogaster* male and female hybrids. However, the *erecta-melanogaster* and *orena-melanogaster* hybrids were lethal, since female *D. erecta* and *D. orena* germ cells in a *D. melanogaster* ovary failed to form oocytes with the capacity to develop normally. This failure appears to be caused by an altered interaction between the *melanogaster* soma and the *erecta* and *orena* germ lines. Germ cells of *D. teissieri* and *D. orena* in a *D. melanogaster* testis produced motile sperm that was not stored in *D. melanogaster* females. This might be due to incompatibility between the *teissieri* and *orena* sperm and the *melanogaster* seminal fluid. A morphological analysis of the terminalia of *yakuba-melanogaster* and *teissieri-melanogaster* hybrids was performed. The effect on the terminalia of *teissieri-melanogaster* hybrids of a mutation in *doublesex*, a regulatory gene that controls the development of the terminalia, was also investigated.

SPECIES are defined as Mendelian populations whose individuals share a common gene pool and between which gene exchange is prevented by reproductive isolating mechanisms (RIMs). Speciation refers to the process by which a population that belongs to a given species permanently separates its lineage. Subsequently, two gene pools (species) evolve independently. RIMs are grouped into two broad classes: prezygotic RIMS, which do not allow the formation of hybrid zygotes between different species, and postzygotic RIMs, which reduce viability or fertility of the hybrids. The question arises as to how many genes contribute to reproductive isolation between closely related species? Also, how do these genes interact to cause hybrid inviability or sterility? Is reproductive isolation caused by mutations in conventional genes or is it the result of novel genetic elements, understood as a class of genes, specifically responsible for the generation of RIMs between evolving species? These and other similar evolutionary questions have been addressed through the analysis of interspecific hybrids.

Hybrid sterility has been studied in Drosophila species-pairs whose $F_1$ female hybrids are fertile but whose male hybrids are sterile. The genetic basis of hybrid sterility has been investigated by the classical analysis of individuals of $F_2$ backcrosses or through introgression analyses. It was found that hybrid sterility appears to be of polygenic nature (reviewed in WU and PALOPOLI 1994). Discrepancies do exist, however, with respect to the genetic organization of these polygenic systems, *i.e.*, whether they are composed of either major- or minor-effect genes. Hybrid sterility was interpreted as a consequence of disrupting co-adapted gene complexes. In the above analysis, it was also found that certain recombinant hybrids were inviable. It has been claimed that the *X* chromosome is mainly responsible for causing hybrid sterility and inviability. However, it has recently been found that there is no evidence to support a strong *X* chromosome bias over autosomal effects in the evolution of hybrid sterility and inviability (HOLLOCHER and WU 1996). The results support the idea that HALDANE's (1922) rule (the heterogametic sex is preferentially affected in interspecific hybrids) actually represents a composite phenomenon (ORR 1993; WU and DAVIS 1993).

Due to the fact that in most cases both male and female interspecific hybrids are sterile, the analysis cannot be taken beyond the first generation. This has been partly overcome, in the case of the genetic basis of hybrid inviability, through clonal analysis in viable hybrids. Thus, the cross of *D. melanogaster* females and *D. simulans* males renders viable hybrid females and lethal hybrid males (STURTEVANT 1920). The viability of cellular clones induced by mitotic recombination in *melanogaster-simulans* hybrid females during larval growth has been analyzed. These clones contained a portion of either *melanogaster* or *simulans* genomes in homozygosity in combination with a hybrid *melanogaster-simulans*

*Corresponding author:* Lucas Sánchez, Centro de Investigaciones Biológicas, Velázquez 144, 28006 Madrid, Spain.
E-mail; cibsr6b@fresno.csic.es

genome. It has been found that the bigger the portion of the genome that is homozygous, the less viable is the recombinant hybrid clone (SÁNCHEZ *et al.* 1994). This holds for all the tested chromosome arms that were analyzed and, then, supports the idea of a polygenic basis as the cause of hybrid lethality. The results were interpreted by considering that the two species *D. melanogaster* and *D. simulans* have diverged to such a degree that the absence of part of the genome of one species cannot be substituted by the corresponding part of the genome of the other, probably due to the formation of co-adapted gene complexes in both species following their divergent evolution after speciation. The disruption of the co-adapted gene complexes would cause the lethality of the recombinant hybrid clones. Recently, it was found that *XO* clones, carrying the *D. melanogaster X* chromosome, in otherwise female *melanogaster-simulans* hybrids are basically lethal and those that survived were very small (ORR *et al.* 1997). This holds also for the *XO* clones induced in female *melanogaster-mauritiana* and *melanogaster-sechellia* hybrids (ORR *et al.* 1997). The analysis of larval neuroblasts revealed that the lethal male *melanogaster-simulans* hybrids suffer a profound mitotic defect characterized by a near-complete failure to condense chromosomes (ORR *et al.* 1997).

The barriers to gene flow between species, if not necessarily a cause of speciation, are at least necessary for maintenance of species identity. Ultimately, these barriers arise as a consequence of genetic diversity among species. The degree of genetic diversity between two species that have evolved from a common ancestor can be measured by comparing protein variation or DNA sequences of single genes. This is a partial estimation of the degree of genetic divergence between the two species, since organisms are not a mere aggregation of genes. They are made of coherent and coordinated genetic complexes. Therefore, an additional and important way of gathering information about genetic diversity between two species is to put the genome of both species together in a cell and see how they interact and cooperate to give rise to hybrids and/or functional gametes. In this respect, two processes are pertinent for the results presented here. Both male and female gametogenesis and the elaboration of maternal morphogenetic information deposited in the oocyte during oogenesis require the interaction of the germ line (oocyte) and the gonadal somatic tissues (follicle cells) (ST. JOHNSTON and NÜSSLEIN-VOLHARD 1992; RAY and SCHÜPBACH 1996). Transplantation of germ cells between species allows the investigation of the capacity of the soma of one species to cooperate with the germ line of another in the production of functional gametes with the capacity to form zygotes which undergo normal development (SANTAMARIA 1977; SÁNCHEZ and SCHMID 1984; SCHMID *et al.* 1984; LAWRENCE *et al.* 1993).

When *D. melanogaster* females are mated with two males, most of their progeny derive from the second male, as a consequence of the sperm from the first male being replaced by the sperm of the second (last-male advantage) (BIRKHEAD 1996). It has been shown that seminal fluid, without sperm, reduces the competitive ability of sperm from other males and incapacitates stored sperm (which is effectively lost), thereby increasing male fitness (HARSHMAN and PROUT 1994). This indicates that a coevolutionary process between sperm and seminal fluid is in action. With the present experimental approach, if sperm from a donor species is produced in the soma of a host species, this would allow the study of the compatibility of the sperm of one species (donor species) and the seminal fluid produced by the accessory glands of the host species. Both sperm and seminal fluid will be transmitted together to the females during copulation of the chimeric males.

Evolutionary changes in morphology do not usually involve the generation of novel cell types but rather changes in morphogenesis; *i.e.*, in the processes that are responsible for the generation of three-dimensional arrangements of various cell types (RAFF and KAUFFMAN 1983). What is the genetic basis for morphological evolution? This question has been also addressed through the analysis of interspecific hybrids. However, analysis has been focused on the effect of quantitative trait loci (QTL), in the different shape of particular structures of a given adult pattern (*e.g.*, COYNE 1983; COYNE *et al.* 1991; LIU *et al.* 1996). Evidence has been found for a polygenic basis of these QTLs. These analyses have been limited to species-pairs that produce fertile females, in order that classical analysis of individuals of $F_2$ backcrosses could be performed. Analysis of morphological variation in other interspecific hybrids cannot be carried out because, in the majority of cases, the species never interbreed, due to ethological isolation or other causes. A way of circumventing this problem is to transplant germ cells between species. If the germ cells from the donor species can develop in the soma of a host species, this may lead to the possibility of producing interspecific hybrids when the chimeric adult hosts are mated with individuals of their own species.

The present analysis focused on the species that form the melanogaster subgroup. This has been divided into three complexes (LACHAISE *et al.* 1988). The melanogaster complex consists of *D. melanogaster, D. simulans, D. mauritiana* and *D. sechellia.* The yakuba complex consists of *D. yakuba* and *D. teissieri*; and the erecta complex is formed by *D. erecta* and *D. orena.* The proposed phylogenetical relationships between these species is shown in Figure 1. It is believed that the first radiation gave rise to the erecta complex. The yakuba complex lineage was subsequently separated from the melanogaster complex (LEE and WATANABE 1987; LACHAISE *et al.* 1988). The production of interspecific hybrids between all of the species that form the melanogaster complex has been reported (LEE and WATANABE 1987). No hybrids have been obtained between the species of the yakuba or the erecta complexes nor between species of the three complexes with the following exceptions: interspecific

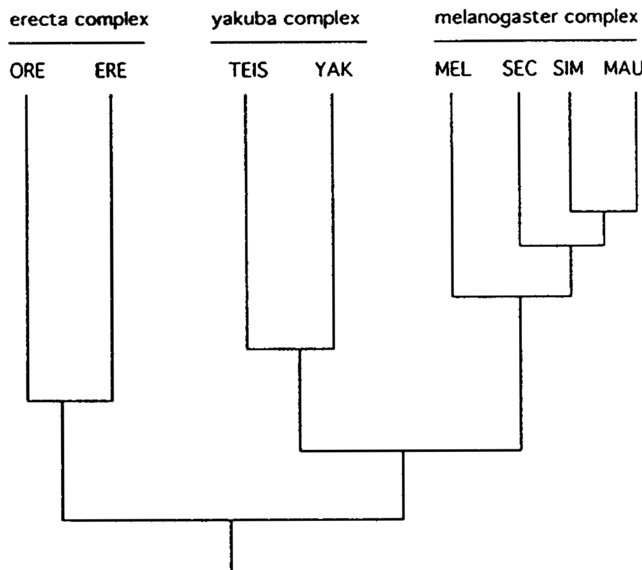



FIGURE 1.—Cladogram showing the phylogenetic relationships among the species of the melanogaster subgroup (LACHAISE *et al.* 1988).

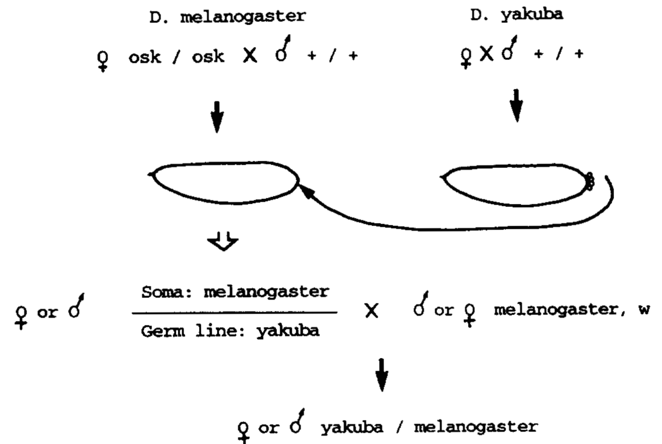



FIGURE 2.—Scheme showing the pole cell transplantation procedure followed in order to produce chimeric flies and the crosses to generate interspecific hybrids.

hybrids have been obtained between *D. mauritiana* and the species of the yakuba and erecta complexes (except for *D. orena*), but only when the males were *D. mauritiana*; and interspecific hybrids have been also reported between *D. simulans* females and *D. teissieri* males, but not in the reciprocal cross (LEE and WATANABE 1987).

This article reports the breaking of ethological barriers through the constitution of soma-germ line chimeras between *D. melanogaster* and the four species that form the yakuba and erecta complexes. This experimental approach allows the study of different RIMs operating in the same species-pairs, rather than analyzing them independently of each other in different species-pairs (till now the most common procedure). An analysis was made of the capacity of germ cells of the species of the yakuba and erecta complexes to form functional gametes in *D. melanogaster* gonads. The viability, fertility and morphology of the terminalia of interspecific hybrids between *D. melanogaster* and the four donor species were also made. In addition, the compatibility of seminal fluid from *D. melanogaster* and sperm from the donor species was evaluated. Further, the effect in the hybrids of a mutation in a regulatory gene that controls the development of the terminalia was also investigated. Different levels of reproductive isolation were observed, which might be the consequence of changes in coadapted gene complexes.

## MATERIALS AND METHODS

Flies were cultured on standard food at 25°. For description of mutations and chromosomes see LINDSLEY and ZIMM (1992).

**Transplantation of pole cells:** Pole cells were transplanted according to the SANTAMARIA (1986) procedure. To generate the host embryos, $p^p$ $osk^{301}$ $e^s$ homozygous females were crossed with *Canton-S* males. All embryos from this cross lacked their own pole cells because they come from mothers homozygous for the *oskar* (*osk*) mutation (LEHMAN and NÜSSLEIN-VOLHARD 1986) (see Figure 2). The transplantation of pole cells was carried out at 18° and the injected embryos were kept at this temperature until the larvae hatched. They were then transferred to vials with Drosophila food and cultured at 25° for the rest of their development. The adult hosts were individually test-crossed with either *D. melanogaster* females or males homozygous for the *white* (*w*) mutation. In the case of chimeric females, this allowed the identification of the expected normal hybrid males carrying the *X* chromosome of the donor species. These showed the dark-red eyes phenotype. The putative patroclinous hybrid males carrying the *D. melanogaster X, w* chromosome showed the white eyes phenotype. In the case of chimeric males, the normal hybrid males showed white eye phenotype, corresponding to the presence of the *D. melanogaster X, w* chromosome, whereas putative patroclinous hybrid males showed dark-red eye phenotype due to the presence of the *X* chromosome of the donor species.

**Analysis of the sterility of chimeric males:** Each adult host male was mated with *D. melanogaster* females (single crosses were performed). As a control, single crosses between *D. melanogaster* females and males were performed. In both experimental and control crosses, the females were sisters of the same age. Copulation was directly observed. After copulation, each female was isolated and subsequently dissected to check for the presence of sperm in the uterus and/or vagina. If sperm was not found, the male was dissected and the testes analyzed to check whether the donor germ cells were integrated in the host testis and, if so, whether they had started to develop, at what stage development was arrested or if the tissue had degenerated. If sperm was found in the females, then the chimeric male was kept for further analysis. This consisted of crossing these males with *D. melanogaster* females (single crosses were performed). As a control, single crosses between *D. melanogaster* females and males were performed. In both experimental and control crosses, the females were sisters of the same age. Copulation was directly observed. After copulation, each female was isolated and kept for either 7 or 24 hr before being dissected to check for the presence of stored sperm in the spermathecae and/or the seminal receptacle. The dissection of the adults was carried out in Ringers solution.

**Analysis of the external terminalia of hybrids:** Flies were kept in a mixture of ethanol/glycerol (3:1) for several days. Afterward, they were macerated in 10% KOH at 60° for 15 min, thoroughly washed with $H_2O$, and mounted in Faures

TABLE 1

**Transplantation of germ cells from species of the yakuba and orena complexes into *D. melanogaster* species**

| Host species | Donor species | No. of injected embryos | No. of adult hosts | | No. of adult hosts that produced interspecific hybrids when crossed with *D. melanogaster* flies | |
|---|---|---|---|---|---|---|
| | | | Females | Males | Females | Males[a] |
| *D. melanogaster* | *D. yakuba* | 379 | 47 | 40 | 3 | 0 |
| *D. melanogaster* | *D. teissieri* | 2359 | 239 | 306 | 15 | 0 |
| *D. melanogaster* | *D. erecta* | 2672 | 163 | 206 | 18 | 0 |
| *D. melanogaster* | *D. orena* | 1070 | 53 | 79 | 5 | 0 |

[a] None of the male hosts produced descendants, although some of them were chimeric and contained male donor germ cells that developed into motile sperm (see text for further explanation).

solution for inspection under a compound microscope. To generate the intersexual *teissieri-melanogaster* hybrids, chimeric *D. melanogaster* females carrying *D. teissieri* germ cells were crossed with *D. melanogaster* males of genotype $y/B^SY$; $dsx^D$ *Sb* $e^s$/*TM6*. The intersexual hybrids were recognized by their Stubble phenotype due to the dominant *Sb* mutation located in the chromosome carrying the $dsx^D$ mutation.

**Analysis of hybrid embryos and larvae:** Embryos were DAPI stained and the cuticle of larvae were prepared following standard procedures (Ashburner 1989).

## RESULTS

**Interspecific chimeras between *D. melanogaster* and the species of the yakuba and erecta complexes:** Since only in *D. melanogaster* species the genetic tools exist to produce embryos lacking their own pole cells (the precursors of the germ line), this species was used as the host in the pole cell transplantation experiments reported here. A comparison was made of the capacity of the germ cells from the four donor species that form the yakuba and erecta complexes to interact with the soma of *D. melanogaster* in the making of functional gametes. Further, by crossing the chimeric adults with males and females of *D. melanogaster*, an analysis was made of the capacity of this species to produce interspecific hybrids with species of the yakuba and erecta complexes. The results are shown in Table 1.

*Donor: D. yakuba:* Of 47 adult host females, three were fertile and produced *yakuba-melanogaster* interspecific hybrids. None of the 40 adult host males produced offspring. The three chimeric females gave rise to female and male hybrids: 97 females and 82 males, 20 females and 19 males and 80 females and 35 males, respectively. None of these interspecific hybrids were fertile. All had rudimentary gonads, similar in phenotype to the gonads of *D. melanogaster* flies carrying mutations that prevent the development of the germ cells. The apparent lack of male chimeras in this and other experiments will be discussed later.

*Donor: D. teissieri:* Of 239 adult host females, 15 were fertile and produced *teissieri-melanogaster* interspecific hybrids. None of the 306 adult host males gave rise to descendants. All of the chimeric females produced female hybrids (797 females in total) but only four produced male hybrids (17 females and 10 males, 18 females and 16 males, 35 females and 19 males and 97 females and 2 males, respectively). The remaining 11 chimeric females, with the exception of one that produced only nine descendants, gave rise to a large progeny (>50 females each one). None of the *teissieri-melanogaster* interspecific hybrids was fertile. All had rudimentary gonads like those of the *yakuba-melanogaster* hybrids described above.

*Donor: D. erecta:* Of 163 adult host females, 18 were fertile. None of the 206 adult host males produced progeny. The chimeric females laid eggs but none developed into adult hybrids.

*Donor: D. orena:* Of 53 adult host females, five were fertile. None of the 79 adult host males produced descendants. The chimeric females laid eggs but none developed into adults, even into larvae.

The *D. melanogaster* soma provided *D. erecta* and *D. orena* germ cells with functions necessary for oogenesis, including yolk intake. These germ cells in a *D. melanogaster* ovary form oocytes that can be fertilized by *D. melanogaster* sperm. However, the development of these *erecta-melanogaster* and *orena-melanogaster* hybrid zygotes was arrested in early development. Of a sample of 408 *erecta-melanogaster* eggs, 287 remained white, 109 turned brown and 12 developed into larvae that hatched, but these could hardly move or eat and died soon after. These eggs were DAPI stained. They showed a nonhomogeneous distribution of syncytial nuclei that, in addition, could form aggregates. The eggs that turned brown represented hybrid zygotes that developed further than the blastoderm stage. Some formed larvae with different degrees of differentiation but none were normal. In the majority of cases, these larvae showed very poorly developed terminal anterior and posterior structures. Occasionally, the main trunk also showed an altered segmentation pattern. Not all the ventral denticle belts corresponding to the different segments were always present. In some cases they were fused. The dorsal hair pattern showed deletions or was completely absent.

In the case of *orena-melanogaster* eggs, of a sample of 257 eggs, 28 turned brown but only in some regions; 229 remained white. Of these, 35 were smaller than normal *melanogaster* or *orena* eggs. None of the eggs gave rise to larvae. As in the *erecta-melanogaster* hybrids, the white *orena-melanogaster* eggs represented hybrid zygotes that did not reach the blastoderm stage and showed a nonhomogeneous distribution of syncytial nuclei. The eggs that were partially brown represented zygotes that showed a certain degree of differentiation but in no case were clear larval structures formed. Furthermore, *orena-melanogaster* chimeric females laid some eggs that were smaller and soft looking. They also showed abnormal chorion and were very easily broken when touched. The chorion is secreted by follicle cells (soma) around the oocyte-nurse cell complex (germ line) and is the result of the interaction between these two cell types (MAHOWALD and KAMBYSELLIS 1980; SCHMID *et al.* 1984; LAWRENCE *et al.* 1993).

In conclusion, the hybrid zygotes from *D. melanogaster* sperm and the yakuba complex species oocytes are viable, whereas those from *D. melanogaster* sperm and the erecta complex species oocytes are lethal.

**Germ cells from species of the yakuba and erecta complexes in a *D. melanogaster* testis produce motile sperm that is not stored in *D. melanogaster* females:** The experimental germ cell transplantation method used was based on the use of host embryos that lack their own pole cells. The production of gametes by these hosts necessarily indicates that the donor germ cells develop into either oocytes or sperm in female and male hosts, respectively. The identification of chimeric female hosts was straightforward since they lay eggs. The identification of chimeric male hosts necessarily requires the analysis of their female partners and their eventual offspring.

None of the *D. melanogaster* males that were injected with germ cells of any of the four donor species, *D. yakuba, D. teissieri, D. erecta* and *D. orena*, produced progeny when crossed with *D. melanogaster* tester females (see Table 1). The sterility of these chimeric males cannot be explained by technical pole cell transplantation failure since chimeric females were obtained nor can it be attributed to intrinsic causes such as the *osk* mutation (LEHMAN and NÜSSLEIN-VOLHARD 1986), as male and female chimeras have been obtained in other experiments (STEINMANN-ZWICKY *et al.* 1989; GRANADINO *et al.* 1993). Therefore, this sterility could be due either to the lack of success of the donor male germ cells to integrate into *D. melanogaster* testes or to their incapacity to produce functional sperm. To analyze the origin of the sterility of the putative chimeric males, we followed the experimental procedure described in MATERIALS AND METHODS. This analysis was carried out for *teissieri-melanogaster* and *orena-melanogaster* chimeric males as representatives of the two phylogenetic lineages to which the four donor species analyzed in this report belong.

Of 306 *teissieri-melanogaster* and 79 *orena-melanogaster* male hosts, 31 and 13, respectively, produced motile sperm that was ejaculated into *D. melanogaster* female partners during copulation. The rest of both types of males had the characteristic rudimentary agametic testes of males with *osk* homozygous mothers. Therefore, it may be concluded that both *D. teissieri* and *D. orena* germ cells can develop in a *D. melanogaster* testis and produce motile sperm whose motility is indistinguishable from that of *D. melanogaster* sperm. The question that arises is why this motile sperm does not give rise to progeny. One possibility is that this sperm, though motile, does not have the capacity to fertilize the *D. melanogaster* oocyte. Another possibility, though not mutually exclusive, is that this sperm might not be stored by *D. melanogaster* females. Each chimeric male was therefore mated with a single *D. melanogaster* female (experimental cross). As a control, single crosses between *D. melanogaster* females and males were performed (see MATERIALS AND METHODS). After copulation the females were isolated and 7 hr later were dissected. In the control crosses, all of the females ($n = 11$) had their seminal receptacles and the two spermathacae full of very active sperm. No sperm was found in the uterus or vagina. In the experimental crosses, 11 of 13 females in the case of *teissieri-melanogaster* chimeric males, and also 11 of 13 females in the case of *orena-melanogaster* chimeric males, showed their seminal receptacles and the two spermathecae to contain no sperm. These exceptional females showed a few spermatozoa in the seminal receptacle. However, the sperm was either nonmotile or its motility was clearly reduced. In addition, all exceptional females had empty spermathecae. Some of the experimental females showed clumps of nonmotile sperm in their uterus and/or vagina. When females were isolated for 24 hr (instead of 7 hr) after copulation and before dissection, none of the experimental females showed sperm in the seminal receptacle nor in the spermathecae. Neither was sperm observed in the uterus and/or vagina. The control females, however, had their seminal receptacle and the two spermathecae full of very motile sperm (data not shown). Therefore, the *D. melanogaster* soma can support the development of *D. teissieri* and *D. orena* male germ cells to produce motile sperm. This sperm, however, cannot be stored by *D. melanogaster* females.

**Morphological studies of the external terminalia of *yakuba-melanogaster* and *teissieri-melanogaster* interspecific hybrids:** The terminalia are produced by a single imaginal disc called the genital disc. At the blastoderm stage, the anlage of the genital disc of both sexes consists of three primordia: the female and male genital primordia (from abdominal segments A8 and A9, respectively), plus the anal primordium (from abdominal segments A10–A11) (NÖTHIGER *et al.* 1977; SCHÜPBACH *et al.* 1978). In every individual, only one of the two genital primordia will grow depending on its genetic sex. The genital primordium that does not develop has

been named the "repressed genital primordium." The anal primordium grows in both sexes.

Female external terminalia are very similar in the three species *D. melanogaster*, *D. yakuba* and *D. teissieri*. The external male terminalia, however, display great differences; indeed, it is one of the main features used in taxonomy (for a description of these species see TSACAS and BOCQUET 1976). The morphology of the external terminalia of *yakuba-melanogaster* and *teissieri-melanogaster* males varied between these two classes of hybrids and their parental species, but was constant for all males belonging to each hybrid class (Figure 3). The morphological differences of the male external terminalia of these hybrids and their parental species could be ascribed to four main categories. (1) There were structures in the three species that showed a different shape in each and were intermediate in shape in their hybrids; for example, the edeagus, the hypandrium and the lateral lobe. (2) There were structures that in the hybrids showed the characteristic shape of one of the parental species; for example, the dorsal parameres in *teissieri-melanogaster* resembled those of *D. teissieri*. (3) There were structures present in the hybrids and in only one of the parental species; *e.g.*, the penis mantle was absent in *D. teissieri* and present in *D. melanogaster* and in *teissieri-melanogaster* hybrids. *D. yakuba* lacked the ventral parameres but these were present in the *yakuba-melanogaster* hybrids. (4) There were structures absent in the hybrids but present in the two parental species; *e.g.*, the ventral parameres were present in *D. melanogaster* and *D. teissieri* but absent in their hybrids.

**Morphological studies of intersexual *teissieri-melanogaster* terminalia:** The sexually dimorphic development of the genital disc is controlled by the sex-determination genes (BAKER 1989; STEINMANN-ZWICKY *et al.* 1990). A hierarchical interaction exists between these genes. The last gene in this hierarchy is *doublesex* (*dsx*). This gene is transcribed in both sexes but its primary transcript follows alternative sex-specific splicing pathways that result in the production of male-specific ($DSX^M$) and female-specific ($DSX^F$) products (BURTIS and BAKER 1989; HOSHIJIMA *et al.* 1991). In the case of the genital disc, genetic data are compatible with the idea that $DSX^M$ and $DSX^F$ control the expression of two sets of genes, one set responsible for the male and the other for the female phenotypes, which would be expressed in the genital disc unless they are repressed by the DSX products. The *doublesex-dominant* ($dsx^D$) mutations transform females into intersexes while having no effect on males (STEIMANN-ZWICKY 1988; BAKER 1989). The *XX*; $dsx^D/+$ intersexual flies contain the $DSX^M$ (from the $dsx^D$ allele) and the $DSX^F$ (from the $dsx^+$ allele) products (NAGOSHI and BAKER 1990). Since the intersexual phenotype of *XX*; $dsx^D/+$ flies is indistinguishable from that produced by loss-of-function *dsx* mutations, the simultaneous presence or absence of $DSX^M$ and $DSX^F$ products within a cell has the same phenotypic effect on the development of the genital disc as if $DSX^M$ and $DSX^F$ counteracted each others function, allowing the simultaneous expression of both male and female sets of genes.

The most conspicuous feature of the intersexual terminalia of *XX*; $dsx^D/+$ *melanogaster* flies is the presence of both male and female genital structures (GOWEN 1942; DENELL and JACKSON 1972; EPPER 1981). These are reduced and arranged in two separate genitalia. Usually, the vagina encloses a small duplication containing rudimentary structures of the penis apparatus. Some intersexual flies show a reduction of genital structures to such an extent that only traces of male and/or female genitalia are present, but these are rare exceptions. The anal plates are always present and their shape resembles neither the wild-type male nor the normal female in appearance. Rather, they display an intermediate sexual phenotype (Figure 4).

The $dsx^D$ allele was introduced into the hybrid via *D. melanogaster* fathers (see MATERIALS AND METHODS). The most salient characteristic of the intersexual terminalia of *XX*; $dsx^D/+$ *teissieri-melanogaster* hybrids was the great difference in the inventory of male *vs.* female genital structures. In general terms, the external genitalia of *XX*; $dsx^D/+$ *teissieri-melanogaster* hybrids could be defined as male-like more than intersex (Figure 4). There was an almost completely normal set of male genital structures. Vaginal plates were never found ($n = 17$) with the exception of one case, in which a rudimentary vagina carrying a single thorn bristle was present. The female eighth tergite was not always present and if so was incomplete. The penis apparatus, though incomplete, was much closer to normality, in terms of inventory of structures and degree of differentiation, than that of *XX*; $dsx^D/+$ *melanogaster* intersexes. The rudimentary duplicated penis was never present. The analia was intersex.

Another important difference between the *melanogaster* and *melanogaster-teissieri* intersexes concerned the development of the sexually dimorphic abdominal segment A7. In wild-type females, segment A7 gives rise to the seventh tergite, dorsally, and to the seventh sternite, ventrally. Both structures are missing in wild-type males. In *XX*; $dsx^D/+$ *melanogaster* intersexes, the seventh tergite and sternite are present. On the contrary, in *XX*; $dsx^D/+$ *teissieri-melanogaster* intersexes, the seventh sternite was absent and the seventh tergite was present in some cases but it was rudimentary (data not shown). Thus, in the intersexual hybrids, the $dsx^D$ mutation gives a more male developmental pathway to the abdominal segment A7 than it does in *melanogaster* intersexes.

## DISCUSSION

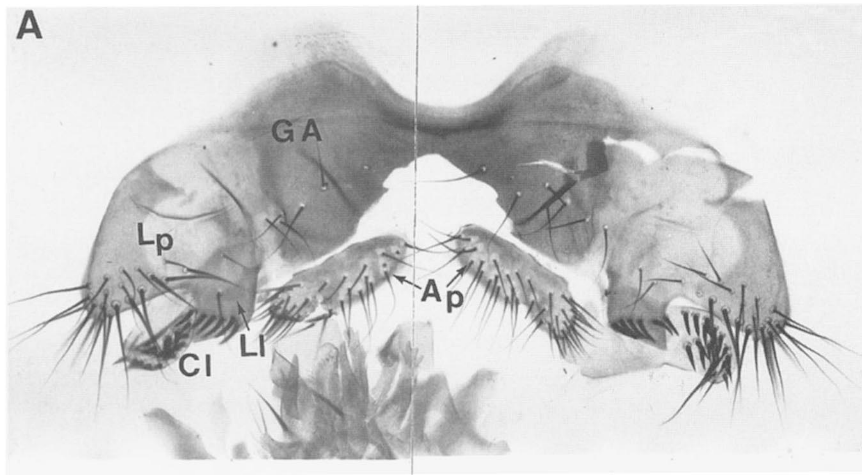
**Viability pattern of hybrids between *D. melanogaster* and the species of yakuba and erecta complexes:** The cross between *D. simulans* females and *D. melanogaster* males yields viable male hybrids and lethal females. Viable females are rare exceptions. Hybrid females die as embryos. The reciprocal cross leads only to female hy-

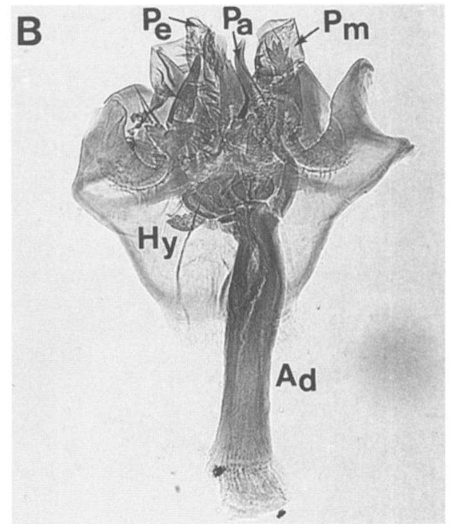
brids; the male hybrids die as third instar larvae (STURTEVANT 1920; LACHAISE *et al.* 1986; LEMEUNIER *et al.* 1986; SAWAMURA *et al.* 1993a,b). The same pattern of interspecific hybrid progeny is obtained when *D. melanogaster* is crossed with the other two sibling species that form the melanogaster complex; namely, *D. mauritiana* and *D. sechellia* (LACHAISE *et al.* 1986; LEMEUNIER *et al.* 1986). The cross between *D. yakuba* females and *D. mauritiana* males yields male and female sterile hybrids (LEE and WATANABE 1987). The same pattern of interspecific hybrid progeny between *D. yakuba* and *D. melanogaster* has been found in the present study since male and female hybrids were found when *D. yakuba* oocytes (from the *yakuba-melanogaster* chimeric females) were fertilized by *D. melanogaster* sperm. The cross between *D. teissieri* females and *D. mauritiana* males yields female and male sterile hybrids (LEE and WATANABE 1987). In the present study, *D. teissieri* oocytes (from *teissieri-melanogaster* chimeric females) that were fertilized by *D. melanogaster* sperm always gave rise to sterile hybrid females and, occasionally, to sterile hybrid males. This occasional absence of male hybrids cannot be ascribed to sampling errors in the progeny size (see RESULTS) but rather is of biological origin. Genetic variation in gene(s) of *D. teissieri* (with maternal or zygotic effect) or in *D. melanogaster* (with zygotic effect) might explain the differences observed in the viability of the *teissieri-melanogaster* hybrid males from different *teissieri-melanogaster* chimeric females. Moreover, it was seen that both *D. erecta* and *D. orena* oocytes (from *erecta-melanogaster* and *orena-melanogaster* chimeric females) fertilized by *D. melanogaster* sperm did not produce viable hybrid zygotes. All these results suggest that hybrid lethality within the melanogaster complex species might have a different cause from that of hybrid lethality between the species of the melanogaster and either yakuba or erecta complexes. Furthermore, the results of the present study are in agreement with the idea that the larger the phylogenetical distance between the species, the lower the capacity of the interspecific hybrid zygotes to develop, and the lower the capacity of germ cells from one species to develop normally in the gonads of another. This might be explained by the disruption of coadapted gene complexes in the different species following their divergent evolution. The results of the present study would fit with the proposed evolutionary tree for the species of the melanogaster subgroup of Figure 1.

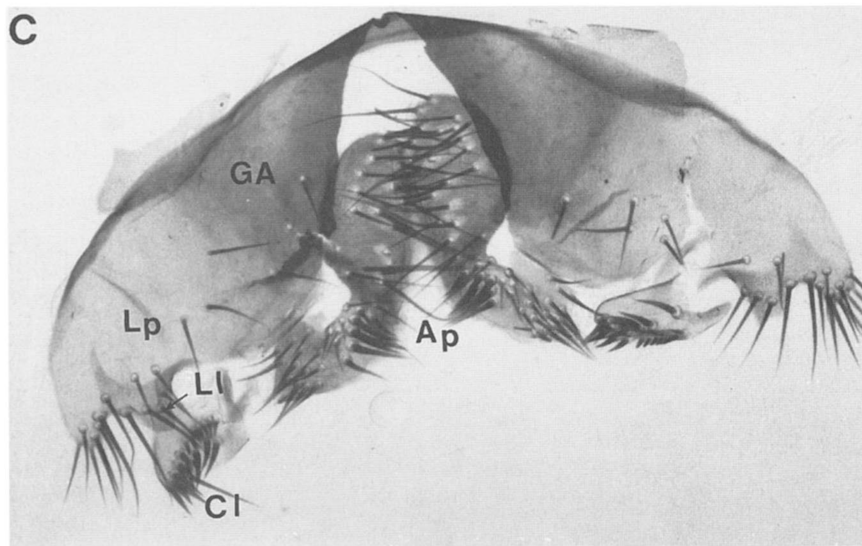
**Altered interaction between *melanogaster* follicle cells and *erecta* or *orena* germ cells:** Germ cells of *D. erecta* and *D. orena* in a *D. melanogaster* ovary form oocytes that can be fertilized by *D. melanogaster* sperm. However, the development of these *erecta-melanogaster* and *orena-melanogaster* hybrid zygotes was arrested in early development. The great majority of *erecta-melanogaster* eggs showed a nonhomogeneous distribution of syncytial nuclei, and the few that developed further than the blastoderm stage formed larvae with very poorly developed terminal anterior and posterior structures and alterations in the segmented and dorsal patterns. In the case of the *orena-melanogaster* hybrid zygotes, the developmental alterations were even more dramatic. It was not easy to discriminate between the maternal deleterious effect of the soma-germ line interaction in the chimera and the incompatibility of the *erecta-melanogaster* and *orena-melanogaster* genomes in the hybrid zygote. However, the phenotype shown by these lethal hybrid zygotes suggests that this lethality is due to the abnormal oocytes produced by the chimeric females as a consequence of a disrupted interaction between the *erecta* or *orena* germ cells and *melanogaster* follicle cells during oogenesis. A similar phenomenon might occur when female *D. rajasekari* germ cells are transplanted into *D. melanogaster* hosts (LAWRENCE *et al.* 1993).

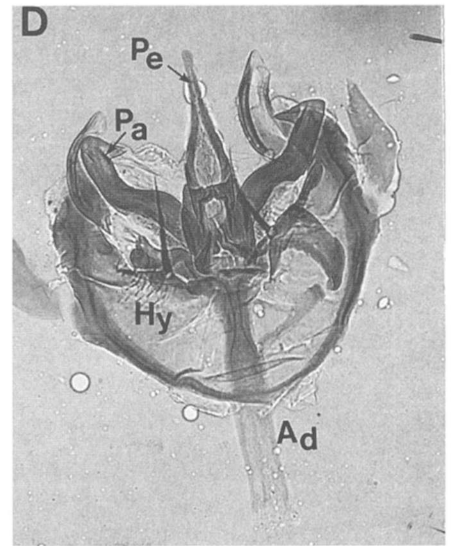
It has been reported that inhibitors of actin filaments disrupt nuclear spacing and chromosome segregation during syncytial divisions (MILLER 1995). Microtubule assembly inhibitors also block cortical nuclear migration and the migrating nuclei are linked by an interdigiting network of microtubules that form aggregates (FOE and ALBERTS 1983; KARR and ALBERTS 1986). The nuclear migration pattern is not inhibited by the injection of $\alpha$-amanitin (EDGAR *et al.* 1986; BAKER *et al.* 1993). This indicates that zygotic transcription is not required in the regulation of nuclear migration and that the cytoskeletal organization of the egg involved in this migration is of maternal origin. The cytoskeletal organization of the oocyte is required for the specific localization of maternal RNAs from genes such as *bicoid* and *nanos* that participate in the generation of anterior-posterior polarity. Furthermore, signals between the developing oocyte and the surrounding follicle cells are required to define the terminal regions and the dorsal-ventral axis of the egg chamber and of the future embryo (RAY *et al.* 1991; ST. JOHNSTON and NÜSSLEIN-VOLHARD 1992; KNOWLES and COOLEY 1994; POKRYWKA 1995). During midoogenesis, microtubule nucleation centers that are present at the posterior end of the oocyte disappear; instead microtubule nucleation centers are established at the anterior end of the oocyte. This process, which depends on signals from the follicle cells, precedes the movement of the oocyte nucleus from the posterior end to an anterior-dorsal cortical position. Then, the nucleus sends signals to the nearby follicle cells, which respond by acquiring a dorsal identity.

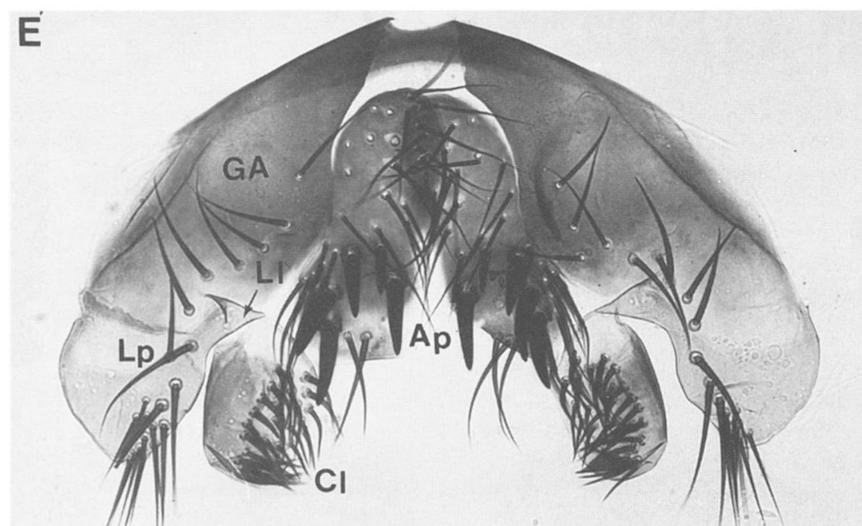
Based on these data, it can be reasoned that the postulated, altered interaction between *erecta* or *orena* germ cells and *melanogaster* follicle cells might prevent normal cytoskeletal organization in the oocytes. This disorganized cytoskeleton would cause the nonhomogeneous distribution of syncytial nuclei observed in the hybrid zygotes. Furthermore, during oogenesis the normal localization of maternal RNAs involved in the formation of the embryonic spatial signals for the future embryo would also be affected. The formation of anterior and posterior structures, as well as segments, could there-



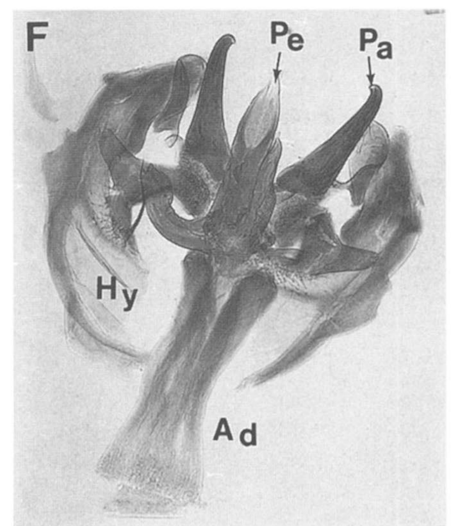




FIGURE 3.—Photographs showing the male external terminalia of *D. melanogaster* (A and B), *D. yakuba* (C and D), *D. teissieri* (E and F), *yakuba-melanogaster* hybrids (G and H) and *teissieri-melanogaster* hybrids (I and J). GA, genital arch; Lp, lateral plate; Ll, lateral lobe; Cl, clasper; Ap, anal plate; Ad, apodeme; Hy, hypandrium; Pe, penis (edeagus), Pa, paramere; Pm, penis mantle. For further description see text.

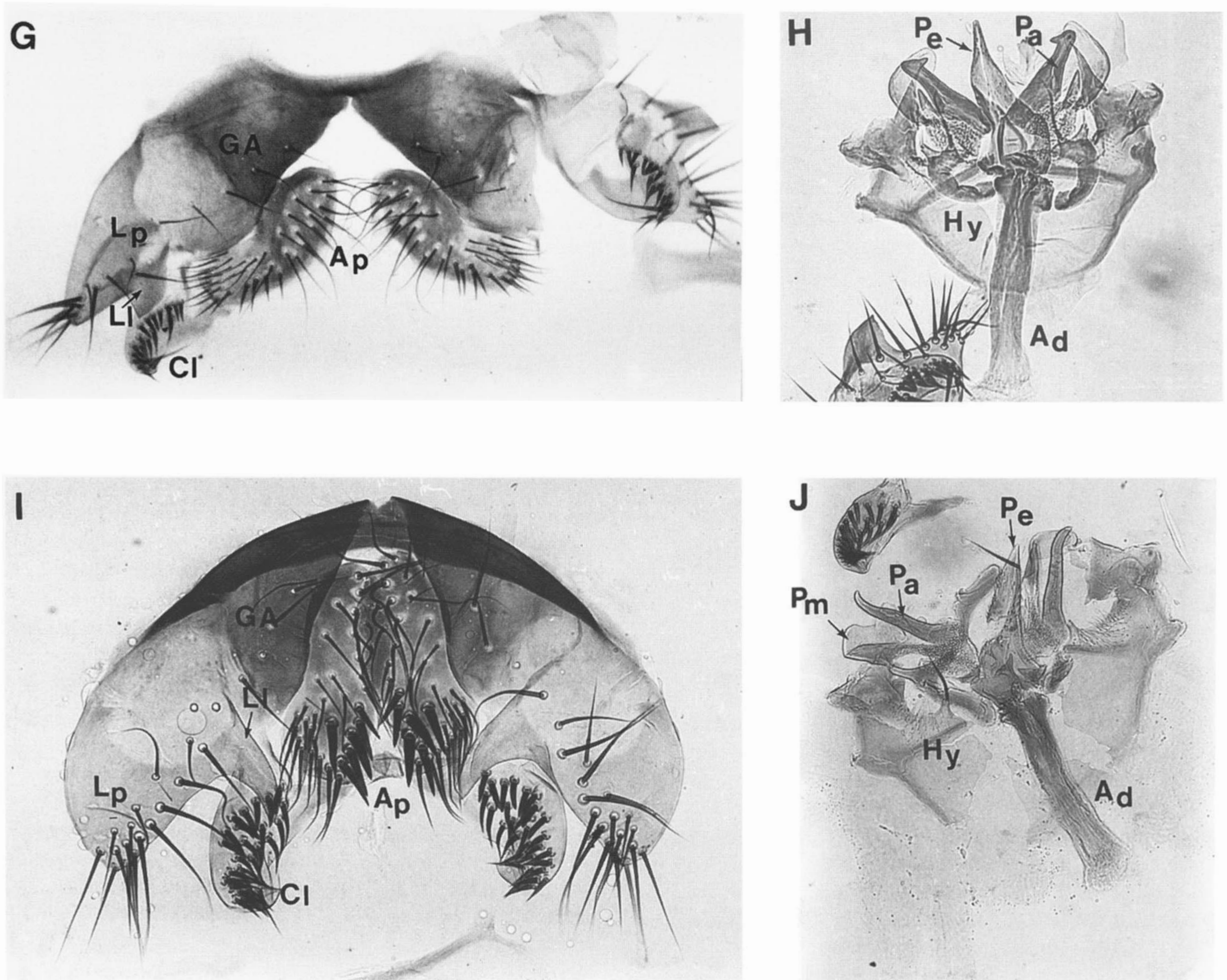



FIGURE 3.—*Continued*

fore be altered. The migration of the oocyte nucleus or signaling between the nucleus and the nearby follicle cells could also be disrupted. Consequently, so too could be the specification of dorsal identity. This could explain the alteration of the dorsal structures seen in the hybrid zygotes.

The failure of female germ cells of *D. erecta* and *D. orena* in a *D. melanogaster* gonad to form oocytes with the capacity to support normal development might be explained by genetic changes that occurred in the coadapted gene complexes responsible for soma-germ line interaction in these species, following their divergent evolution after speciation. Signals from one tissue and their receptors in another might no longer be compatible. It is likely that these genetic changes arose after the phylogenetic lineage of the erecta complex separated from the other phylogenetic lineages that gave rise to the melanogaster and yakuba complexes, since female germ cells of the latter complex can form functional oocytes in a *D. melanogaster* gonad.

**Failure of *teissieri* and *orena* sperm to be stored in *D. melanogaster* females:** Germ cells from *D. teissieri* and *D. orena* in a *D. melanogaster* testis produced motile sperm that is not stored in *D. melanogaster* females. This could be due to a rejection reaction by these females against the *teissieri* and *orena* sperm, which may not be recognized as sperm of its own species. Anti-alien or self-recognition peptides are expressed in the genital tract (HETRU *et al.* 1994; KAPPLER *et al.* 1994; HOFFMAN 1995). Even if their role is mostly antibacterial, they could be the forerunners of a primary immune reaction. Another possibility, not mutually exclusive, is the existence of incompatibility between the sperm produced by the *teissieri* or *orena* germ cells and the seminal fluid synthesized by the *melanogaster* accesory glands of the same males. Both are transmitted together to the females during copulation. It has been shown that seminal fluid, without sperm, reduces the competitive ability of sperm from other males and incapacitates stored sperm (which is effectively lost), thereby increasing male fitness (HARSHMAN and PROUT 1994). This indicates that a coevolutionary process between sperm and

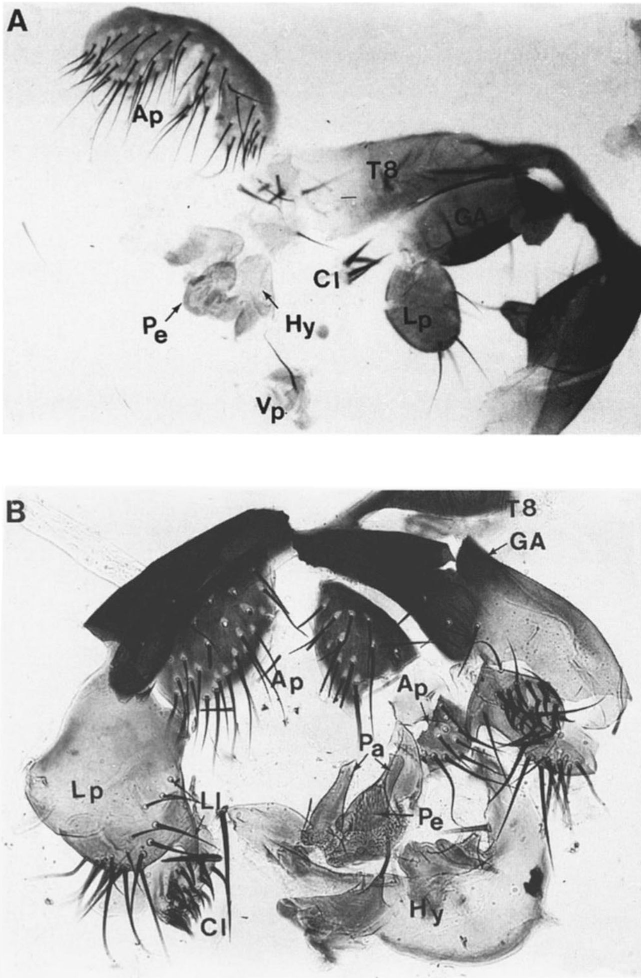



FIGURE 4.—Photographs showing the male external terminalia of *XX; $dsx^D$/+ melanogaster* intersexual flies (A) and *XX; $dsx^D$/+ teissieri-melanogaster* intersexual hybrids (B). Vp, vaginal plate; T8, eight tergite. For the rest of the symbols see Figure 3 legend. For further description see text.

seminal fluid is in action. Studies on polymorphism and divergence in genes producing seminal fluid proteins from species of the melanogaster complex reveal a high degree of variation, indicative of high rates of evolution (AGUADÉ *et al.* 1992). This is inconsistent with a "neutral" theory of molecular evolution and confirms that the seminal fluid proteins are targets of adaptive evolution. In fact, this male adaptation has recently received experimental support. When *D. melanogaster* females are experimentally prevented from coevolving with males, these females become more sensitive to the seminal fluid of separately evolving males, so that this seminal fluid has an increased toxicity for the females (RICE 1996). In the case of the *teissieri-melanogaster* and *orena-melanogaster* chimeric males, the present study placed together *teissieri* and *orena* sperm with *melanogaster* seminal fluid after these species have evolved separately for a long time. It is suggested that the sperm and seminal fluid are no longer compatible. This could explain why the sperm was not stored. The mechanism by which the sperm fails to be stored in females might constitute a RIM between these species.

**Morphological evolution of Drosophila terminalia:** The morphological differences in the external terminalia of *yakuba-melanogaster* and *teissieri-melanogaster* male hybrids and those of their parental species can be either qualitative or quantitative. It is speculated that these morphological differences correspond to changes in two basic genetic mechanisms controlling the generation of morphological patterns. Qualitative differences, which refer to the fact that a given structure of the pattern is either present or absent, probably reflect a simple genetic basis in which a single gene is at work (or a combination of a few genes, each one having a qualitative effect). Quantitative differences, however, are characterized because the structure displays more or less continuous variation, or intermediate phenotype, suggesting that a polygenic system is at work (each gene in the system has a minor effect and shows additivity).

The effect of a regulatory mutation, $dsx^D$, in the development of the *teissieri-melanogaster* terminalia has been also investigated. The different effect of this mutation in *melanogaster* and in *teissieri-melanogaster* flies might be explained in terms of regulatory changes in gene expression that occurred during the evolution of the species *D. melanogaster* and *D. teissieri*. As mentioned above, *dsx* is the last gene in the genetic hierarchy that controls the development of the sexually dimorphic regions of the fly. The gene *dsx* must act in concert with another regulatory gene(s) to determine the genital primordia that will develop in each sex. In females, the abdominal embryonic segment A8 develops to form the female genitalia. In males, the abdominal embryonic segment A9 develops to form the male genitalia (NÖTHIGER *et al.* 1977; SCHÜPBACH *et al.* 1978; EPPER and NÖTHIGER 1982; JÜRGENS and HARTENSTEIN 1993). A possible candidate for this additional regulatory element is the homeotic gene *Abdominal-B* (*Abd-B*), since this gene is responsible for specification of posterior abdominal embryonic segments A7, A8 and A9 (SÁNCHEZ-HERRERO *et al.* 1985; CASANOVA *et al.* 1986; DUNCANV 1987). We cannot eliminate the possibility that *Abd-B* itself was not the additional regulatory gene. It might have been any other gene under its control. It has been argued that changes in homeotic genes (SLACK *et al.* 1993; TABIN and LAUFER 1993; CARROLL 1995) and/or their regulatory genes, such as *Pc-G* genes (SANTAMARIA 1993; WANG *et al.* 1996) play an important role in morphological evolution. Then, species-specific variations in *Abd-B* might also constitute a source for the morphological changes observed in the terminalia of the different species.

It may be speculated that, during the evolution of these species, genetic changes have occurred in the regulatory genes, such as *dsx* and/or *Abd-B*, and/or the genes controlled by these regulators, which are responsible for the development of the terminalia. These species-specific variations would be responsible for the morphological changes observed in the terminalia of these species.

When the genomes of two species are put together within a hybrid cell, divergent co-adapted gene complexes are confronted. This might result in the formation of hybrid patterns different from that of their parental species; *i.e.*, in the production of morphological diversity. This effect would be stronger if the hybrid cell receives an ambiguous regulatory signal, as occurs in the case of *XX; dsx^D/+* intersexes, whose cells contain both male- and female-specific DSX regulatory products.

**Conclusion:** To understand evolution it is crucial to understand how RIMs form during speciation. It is not known whether speciation of the melanogaster, yakuba and erecta complexes was sympatric or allopatric (LACHAISE *et al.* 1988), as we do not know whether it was due to genetic variation in a small number of genes or if this required extensive genetic variation. In this respect, multilocus weak allele interactions (CABOT *et al.* 1994) and epistatic interactions between conspecific genes (DAVIS *et al.* 1994) have been proposed as having important roles as RIMs within the melanogaster complex.

Different levels of reproductive isolation were observed between *D. melanogaster* and the species of the yakuba and erecta complexes. It was found that female germ cells of *D. erecta* and *D. orena* in a *D. melanogaster* gonad failed to form oocytes with the capacity to support normal development. This failure might be explained by genetic changes that occurred in the co-adapted gene complexes responsible for soma-germ line interaction in these species, following their divergent evolution after speciation. It was also found that sperm from the species of the yakuba and erecta complexes cannot be stored by *melanogaster* females. This might be also explained by genetic changes that affected the co-adaptation of sperm and seminal fluid or the vaginal environment. The morphological differences in the external terminalia of these species, as revealed by the comparative analysis of *yakuba-melanogaster* and *teissieri-melanogaster* male hybrids and their parental species, and the different effect in *D. melanogaster* and in *teissieri-melanogaster* hybrids of a mutation in *dsx*, a regulatory gene that controls the development of the genital disc, can be understood in terms of changes in co-adapted gene complexes that govern the morphogenesis of the terminalia. Thus, the different levels of reproductive isolation observed here would constitute examples of how RIMs would arise as secondary effects of genetic changes operating in the co-adapted gene complexes responsible for the development, the physiology and the behavior of the organisms.

We are grateful to D. MATEOS and R. DE ANDRÉS for their technical assistance. This work was supported by grant PB92-0006 from DGICYT to L.S. The Ayuda from Convenio CSIC-CNRS to L.S. and from Programme de Recherche en Cooperation sur Conventions Internationales du CNRS to P.S. is also acknowledged.

## LITERATURE CITED

AGUADÉ, M., N. MIYASHITA and C. H. LANGLEY, 1992 Polymorphism and divergence in the *Mst26A* male accessory gland gene region in Drosophila. Genetics **132:** 755–770.

ASHBURNER, M., 1989 *Drosophila. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

BAKER, B. S., 1989 Sex in flies: the splice of life. Nature **340:** 521–524.

BAKER, J., W. E. THEURKAUF and G. SCHUBIGER, 1993 Dynamic changes in microtubule configuration correlate with nuclear migration in the preblastoderm Drosophila embryo. J. Cell Biol. **122:** 113–121.

BIRKHEAD, T. R., 1996 Sperm competition: evolution and mechanisms. Curr. Top. Dev. Biol. **33:** 103–158.

BURTIS, K. C., and B. S. BAKER, 1989 Drosophila *doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell **56:** 997–1010.

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.

CARROLL, S. B., 1995 Homeotic genes and the evolution of arthropods and chordates. Nature **376:** 479–485.

CASANOVA, J., E. SÁNCHEZ-HERRERO and G. MORATA, 1986 Identification and characterization of a parasegment specific regulatory element of the *Abdominal-B* gene of Drosophila. Cell **47:** 627–636.

COYNE, J. A., 1983 Genetic basis of differences in genital morphology among three sibling species of Drosophila. Evolution **37:** 1101–1118.

COYNE, J. A., J. RUX and J. R. DAVID, 1991 Genetics of morphological differences and hybrid sterility between *Drosophila sechellia* and its relatives. Gent. Res. Camb. **57:** 113–122.

DAVIS, A. W., E. G. NOONBURG and C. I. WU, 1994 Evidence for complex genic interactions between conspecific chromosomes underlying hybrid female sterility in the *Drosophila simulans* clade. Genetics **137:** 191–199.

DENELL, R. E., and J. JACKSON, 1972 A genetic analysis of transformer-Dominant. Dros. Inf. Serv. **48:** 45.

DUNCAN, I., 1987 The bithorax complex. Annu. Rev. Genet. **21:** 285–319.

EDGAR, B. A., C. P. KIEHLE and G. SCHUBIGER, 1986 Cell cycle control by the nucleocytoplasmic ratio in early Drosophila development. Cell **44:** 365–372.

EPPER, F., 1981 Morphological analysis and fate map of the intersexual genital disc of the mutant *doublesex-dominant* in *Drosophila melanogaster.* Dev. Biol. **88:** 104–114.

EPPER, F., and R. NÖTHIGER, 1982 Genetic and developmental evidence for a repressed genital primordium in *Drosophila melanogaster.* Dev. Biol. **94:** 163–175.

FOE, V. E., and B. M. ALBERTS, 1983 Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in Drosophila embryogenesis. J. Cell Sci. **61:** 31–70.

GOWEN, J. W., 1942 Hermaphrodites in *Drosophila melanogaster.* Dros. Inf. Serv. **16:** 63.

GRANADINO, B., P. SANTAMARÍA and L. SÁNCHEZ, 1993 Sex determination in the germ line of *Drosophila melanogaster*: activation of the gene *Sex-lethal.* Development **118:** 813–816.

HALDANE, J. B. S., 1922 Sex ratio and unisexual sterility in hybrid animals. J. Genet. **12:** 101–109.

HARSHMAN, L. G., and T. PROUT, 1994 Sperm displacement without sperm transfer in *Drosophila melanogaster.* Evolution **48:** 758–766.

HETRU, C., P. BULET, C. S, J. L. DIMARCQ, D. HOFFMAN and J. A. HOFFMAN, 1994 Antibacterial peptides-polypeptides in the insect host defense: a comparison with vertebrate antibacterial peptides-polypeptides, pp. 43–65 in *Perspectives in Immunity: The Insect Host Defense,* edited by J. A. HOFFMAN, C. A. JANEWAY and S. NATORI. R. G. Landes Company, Austin, TX.

HOFFMAN, J. A., 1995 Innate immunity of insects. Curr. Opin. Immunol. **7:** 4–10.

HOLLOCHER, H., and C. I. WU, 1996 The genetics of reproductive isolation in the *Drosophila simulans* clade: X *vs.* autosomal effects and male *vs.* female effects. Genetics **143:** 1243–1255.

HOSHIJIMA, K., K. INOUE, I. HIGUCHI, H. SAKAMOTO and Y. SHIMURA, 1991 Control of *doublesex* alternative splicing by *transformer* and *transformer-2* in Drosophila. Science **252:** 833–836.

JÜRGENS, G., and V. HARTENSTEIN, 1993 The terminal regions of the body pattern, pp. 687–746 in *The Development of Drosophila melanogaster,* edited by M. BATE and A. MARTÍNEZ-ARIAS. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

KAPPLER, C., M. LAGUEUX, J. M. REICHHART and J. A. HOFFMAN, 1994 Immune gene expression in Drosophila, pp. 167–181 in *Perspectives in Immunity: The Insect Host Defense*, edited by J. A. HOFFMAN, C. A. JANEWAY and S. NATORI. R. G. Landes Company, Austin, TX.

KARR, T. L., and B. M. ALBERTS, 1986 Organization of the cytoskeleton in early Drosophila embryos. J. Cell Biol. **102:** 1494–1509.

KNOWLES, B. A., and L. COOLEY, 1994 The specialized cytoskeleton of the Drosophila egg chamber. Trends Genet. **10:** 235–241.

LACHAISE, D., J. R. DAVID, F. LEMEUNIER, L. TSACAS and M. ASHBURNER, 1986 The reproductive relationships of *Drosophila sechellia* with *D. mauritiana* and *D. melanogaster* from the Afrotropical region. Evolution **40:** 262–271.

LACHAISE, D., M. L. CARIOU, J. R. DAVID, F. LEMEUNIER, L. TSACAS *et al.*, 1988 Historical biogeography of the *Drosophila melanogaster* species subgroup. Evol. Biol. **22:** 159–225.

LAWRENCE, P. A., M. ASHBURNER and P. JOHNSTON, 1993 An attempt to hybridize Drosophila species using pole cell transplantation. Genetics **134:** 1145–1148.

LEE, W. H., and T. K. WATANABE, 1987 Evolutionary genetics of the *Drosophila melanogaster* subgroup.I. Phylogenetic relationships based on matings, hybrids and proteins. Jpn. J. Genet. **62:** 225–239.

LEHMAN, R., and C. NÜSSLEIN-VOLHARD, 1986 Abdominal segmentation, pole cell formation, and embryonic polarity requires the localized activity of *oskar*, a maternal gene in Drosophila. Cell **47:** 141–152.

LEMEUNIER, F., L. TSACAS, J. R. DAVID and M. ASHBURNER, 1986 The melanogaster species group, pp. 147–256 in *The Genetics and Biology of Drosophila*, edited by M. ASHBURNER, H. L. CARSON and J. N. THOMPSON, JR. Academic Press, London.

LINDSLEY, D. L., and G. ZIMM, 1992 *The Genome of Drosophila melanogaster.* Academic Press, San Diego.

LIU, J., J. M. MERCER, L. F. STAM, G. C. GIBSON, Z. B. ZENG *et al.*, 1996 Genetic analysis of a morphological shape difference in the male genitalia of *Drosophila simulans* and *D. mauritiana.* Genetics **142:** 1129–1145.

MAHOWALD, A. P., and M. P. KAMBYSELLIS, 1980 Oogenesis, pp. 141–224 in *The Genetics and Biology of Drosophila*, edited by M. ASHBURNER and T. R. F. WRIGHT. Academic Press, London.

MILLER, K. C., 1995 Role of the actin cytoskeleton in early Drosophila development. Curr. Top. Dev. Biol. **31:** 167–196.

NAGOSHI, R. N. and B. S. BAKER, 1990 Regulation of sex-specific RNA splicing at the Drosophila *doublesex* gene: *cis*-acting mutations in exon sequences alter sex-specific RNA splicing patterns. Genes Dev. **4:** 89–97.

NÖTHIGER, R., A. DÜBENDORFER and F. EPPER, 1977 Gynandromorphs reveal two separate primordia for male and female genitalia. Roux's Arch. Dev. Biol. **181:** 367–373.

ORR, H. A., 1993 Haldane's rule has multiple genetic causes. Nature **361:** 532–533.

ORR, H. A., L. D. MADDEN, J. A. COYNE, R. GOODWIN and R. S. HAWLEY 1997 The developmental genetics of hybrid inviability: a mitotic defect in Drosophila hybrids. Genetics **145:** 1031–1040.

POKRYWKA, N. J., 1995 RNA localization and the cytoskeleton in Drosophila oocytes. Curr. Top. Dev. Biol. **31:** 139–166.

RAFF, R. A., and T. C. KAUFFMAN, 1983 *Embryos, Genes and Evolution: The Developmental Genetic Basis of Evolutionary Change.* Collier Macmillan Publishers, London.

RAY, R. P., and T. SCHÜPBACH, 1996 Intercellular signaling and the polarization of body axes during Drosophila oogenesis. Genes Dev. **10:** 1711–1723.

RAY, R. P., K. ARORA, C. NÜSSLEIN-VOLHARD and W. M. GELBART, 1991 The control of cell fate along the dorsal-ventral axis of the Drosophila embryo. Development **113:** 35–54.

RICE, W. R., 1996 Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. Nature **381:** 232–234.

SÁNCHEZ, L., and H. SCHMID, 1984 Analysis of the sterility of male hybrids between *Drosophila simulans* and *Drosophila mauritiana.* Genetics **107** (Suppl.): s93–s94.

SÁNCHEZ, L., B. GRANADINO and L. VICENTE, 1994 Clonal analysis in hybrids between *Drosophila melanogaster* and *Drosophila simulans.* Roux's Arch. Dev. Biol. **204:** 112–117.

SÁNCHEZ-HERRERO, E., I. VERNÓS, R. MARCO and G. MORATA, 1985 Genetic organization of Drosophila bithorax complex. Nature **313:** 108–113.

SANTAMARIA, P., 1977 On the causes of sterility in some interspecific hybrids from the melanogaster subgroup of Drosophila. Roux's Arch. Dev. Biol. **182:** 305–310.

SANTAMARIA, P., 1986 Injecting eggs, pp. 159–173, in *Drosophila, A Practical Approach*, edited by O. B. ROBERTS. IRL Press, Arlington, VA.

SANTAMARIA, P., 1993 Evolution and aggregulates: role of the *Polycomb-group* genes of Drosophila. C. R. Acad. Sci. Paris, Science de la vie. **316:** 1200–1206.

SAWAMURA, K., T. TAIRA and T. K. WATANABE, 1993a Hybrid lethal systems in the *Drosophila melanogaster* species complex. I. The *maternal hybrid rescue* (*mhr*) gene of *Drosophila simulans.* Genetics **133:** 299–305.

SAWAMURA, K., M.-T. YAMAMOTO and T. K. WATANABE, 1993b Hybrid lethal systems in the *Drosophila melanogaster* species complex. II. The *Zygotic hybrid rescue* (*Zhr*) gene of *D. melanogaster.* Genetics **133:** 307–313.

SCHMID, H., L. SÁNCHEZ and R. NÖTHIGER, 1984 Heterospecific combinations of germ cells and gonadal soma between *Drosophila melanogaster, D. mauritiana* and *D. ananassae.* Roux's Arch. Dev. Biol. **194:** 99–106.

SCHÜPBACH, T., E. WIESCHAUS and R. NÖTHIGER, 1978 The embryonic organization of the genital disc studied in genetic mosaics of *Drosophila melanogaster.* Roux's Arch. Dev. Biol. **185:** 249–270.

SLACK, J. M. W., P. W. H. HOLLAND and C. F. GRAHAM, 1993 The zootype and the phylotypic stage. Nature **361:** 490–492.

ST. JOHNSTON, D., and C. NÜSSLEIN-VOLHARD, 1992 The origin of pattern and polarity in the Drosophila embryo. Cell **68:** 201–220.

STEIMANN-ZWICKY, M., 1988 Sex determination in Drosophila: the X chromosomal gene *liz* is required for *Sxl* activity. EMBO J. **7:** 3889–3898.

STEINMANN-ZWICKY, M., H. SCHMID and R. NÖTHIGER, 1989 Cell-autonomous and inductive signals can determine the sex of the germ line of Drosophila by regulating the gene *Sxl.* Cell **57:** 157–166.

STEINMANN-ZWICKY, M., H. AMREIN and R. NÖTHIGER, 1990 Genetic control of sex determination in Drosophila, pp. 189–337, in *Advances in Genetics*, edited by T. R. F. WRIGHT. Academic Press, San Diego.

STURTEVANT, A. H., 1920 Genetic studies on *Drosophila simulans.* I. Introduction. Hybrids with *Drosophila melanogaster.* Genetics **5:** 488–500.

TABIN, C., and E. LAUFER, 1993 *Hox* genes and serial homology. Nature **361:** 692–693.

TSACAS, L., and C. BOCQUET, 1976 Lespèce chez les Drosophilidae. Mém. Soc. Zool. Fr. **39:** 203–247.

WANG, D. G., J. L. MARSH and F. J. AYALA, 1996 Evolutionary changes in the expression pattern of a developmentally essential gene in three Drosophila species. Proc. Natl. Acad. Sci. USA **93:** 7103–7107.

WU, C.-I., and A. W. DAVIS, 1993 Evolution of postmating reproductive isolation: the composite nature of Haldane's rule and its genetic basis. Am. Nat. **142:** 187–212.

WU, C.-I., and M. F. PALOPOLI, 1994 Genetics of postmating reproductive isolation in animals. Annu. Rev. Genet. **27:** 283–308.

Communicating editor: T. SCHÜPBACH