The Genetics of Postzygotic Isolation in the *Drosophila virilis* Group

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Manuscript received August 15, 1988
Accepted for publication November 25, 1988

ABSTRACT

In a genetic study of postzygotic reproductive isolation among species of the *Drosophila virilis* group, we find that the *X* chromosome has the largest effect on male and female hybrid sterility and inviability. The *X alone* has a discernible effect on postzygotic isolation between closely related species. Hybridizations involving more distantly related species also show large *X*-effects, although the autosomes may also play a role. In the only hybridization yet subjected to such analysis, we show that hybrid male and female sterility result from the action of *different* *X*-linked loci. Our results accord with genetic studies of other taxa, and support the view that both HALDANE's rule (heterogametic F_1 sterility or inviability) and the large effect of the *X* chromosome on reproductive isolation result from the accumulation by natural selection of partially recessive or underdominant mutations. We also describe a method that allows genetic analysis of reproductive isolation between species that produce completely sterile or inviable hybrids. Such species pairs, which represent the final stage of speciation, cannot be analyzed by traditional methods. The *X* chromosome also plays an important role in postzygotic isolation between these species.

The origin of isolating mechanisms is . . . a problem of fundamental importance, and the paucity of our knowledge on this subject is felt as a glaring defect in the whole doctrine of evolution.

DOBZHANSKY and KOLLER (1938)

BECAUSE it is impossible to observe speciation, understanding the process depends on reconstructing history. It is sometimes possible, for example, to identify the genetic changes causing reproductive isolation by crossing pairs of incipient species. Such studies invariably show that the genes with the largest effect on hybrid sterility and inviability are on the *X* chromosome (data summarized in COYNE and ORR 1989a). While most of this work involves the sterility or inviability of male Drosophila hybrids, other insect species also show a large *X*-effect (COYNE and ORR 1989a). The *X* chromosome also plays a disproportionately large role in the sterility and inviability of hybrid *females*, although few cases have been studied (ORR 1987). CHARLESWORTH, COYNE and BARTON (1987) and COYNE and ORR (1989a) suggest that the large effect of the *X* chromosome on postzygotic isolation results from the rapid divergence of *X*-linked loci by natural selection.

Here we report a study of the genetics of hybrid sterility in seven species of the *Drosophila virilis* group (THROCKMORTON 1982). The phylogeny of this group, which includes 12 species, is well-established because electrophoretic, karyotypic, and morphological data give consistent patterns of relatedness [THROCKMORTON (1982), and unpublished data; MACINTYRE and COLLIER (1986)]. Many of the species can be crossed, and much is known about the fertility and viability of the resulting hybrids (PATTERSON and STONE 1952). However, few genetic analyses of postzygotic isolation have been performed in the *virilis* group, and all of these have ignored the *X* chromosome (see PATTERSON and STONE 1952). Inclusion of the sex chromosomes in such studies can help answer several questions:

1. Do the large *X* effect and HALDANE's rule result from the more rapid evolution of the *X* chromosome by natural selection during speciation? CHARLESWORTH, COYNE and BARTON (1987) showed that the large *X*-effect could result from the accumulation of favorable but partially recessive or underdominant mutations during divergence of geographically isolated populations. COYNE and ORR (1989a) further showed that if there is some correlation between the sex in which a mutation is first favored and the hybrid sex it ultimately afflicts, alleles causing postzygotic isolation will cluster on the *X* chromosome *and* those alleles affecting the heterogametic sex will accumulate faster than those affecting the homogametic sex. This difference in substitution rates may explain HALDANE's rule, the preferential sterility or inviability of heterogametic hybrids in species crosses (HALDANE 1922).

These recent theories make at least two testable predictions, both of which differ from those resulting from the only alternative explanation of the *X*-effect and HALDANE's rule, that of MULLER (1940, 1942). MULLER's explanation and the predictions distinguishing these two hypotheses are discussed later (see DISCUSSION).

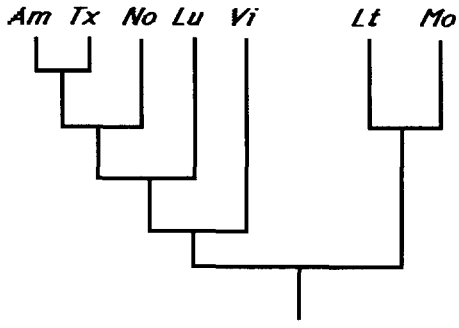

FIGURE 1.—Phylogeny of the *D. virilis* group. This phylogeny is based on concordant electrophoretic, morphological and karyotypic characters (see text). Am = *americana*, Tx = *texana*, No = *novamexicana*, Lu = *lummei*, Vi = *virilis*, Lt = *littoralis*, Mo = *montana*.

2. Is the large *X*-effect an artifact of analyzing only species pairs that produce some viable, fertile hybrids? As COYNE and ORR (1989a) note, *X*-linked genes may be more likely than autosomal genes to affect only one hybrid sex, because *X*-linked recessives are expressed only in hybrid males. There could thus be many species pairs that—because of incompatibilities among the autosomes—produce sterile or inviable hybrids of *both* sexes. But such hybridizations cannot be studied with traditional genetic methods, which require the use of some viable and fertile F_1 hybrids. Thus genetically analyzable species pairs could represent a biased sample of hybridizations, weighted toward those showing large *X*-effects.

To determine if the *X*-effect results from such an empirical bias, we require a way to study the genetics of reproductive isolation in crosses producing sterile or inviable hybrids of *both* sexes. Here we describe such a method. Our procedure is based on the fact that two "uncrossable" species may cross successfully with a third species, yielding viable and fertile hybrids. This third species can thus serve as a bridge to transfer chromosomes between the first two, allowing one to determine the genetic basis of reproductive isolation between the uncrossable species. Here we apply this method to *Drosophila americana* and *Drosophila montana*, species which produce completely sterile F_1 males and females in both reciprocal crosses (THROCKMORTON 1982).

3. Do the same loci cause hybrid sterility or inviability in both sexes? We do not presently know whether hybrid male and female sterility/inviability are caused by the same genes. Such information would be useful for two reasons. First, it might allow better estimates of the total number of loci causing postzygotic isolation; second, it could provide clues about the normal function of these genes (*e.g.*, do they act during meiosis or during the later stages of gametogenesis?).

MATERIALS AND METHODS

The species: THROCKMORTON (1982) summarizes the biology of the *Drosophila virilis* group, which consists of 12 species divided into two phylads: the *virilis* and the *montana*. Both phylads are probably of Asian origin, but some species in each also occur in or are endemic to North America. We used five species in the *virilis* and two in the *montana* phylad (Figure 1). Four of these species have six pairs of chromosomes: the sex chromosomes (chromosome *1*), four pairs of large autosomes (2–5), and one pair of tiny autosomes (6). In *Drosophila texana* and *D. americana*, the second and third chromosomes are fused into a single large autosome, and in the latter species the *X* (but not the *Y*) has also fused with the fourth. *Drosophila littoralis* has a fusion between the third and fourth chromosomes. The two phylads also differ by a pericentric inversion on the second chromosome, which is acrocentric in the *virilis* phylad but metacentric in the *montana*. Crosses within phylads produce fertile offspring much more often than those between phylads, but almost all crosses obey HALDANE's rule.

TABLE 1

Strains used in this study

Drosophila virilis	
white:	A strain constructed by introgressing the Bowling Green *white* eye color allele [map position 1-105; ALEXANDER (1976)] into a wild-type strain of *D. virilis* and reextracting the mutant
yellow, apricot:	*yellow* (*y*) at 1–2.9 and *apricot* (*ap*) at 1–136.0 (ALEXANDER 1976). As the *X* is about 170 map units long, each half is marked with a mutation
D. americana	Myrtle Beach State Park, South Carolina (Bowling Green Strain 15010-0951.90); Red Cloud, Nebraska (obtained from L. THROCKMORTON)
D. lummei	Finland (15010-1011)
D. novamexicana	San Antonia, New Mexico (15010-1031.8)
D. texana	Morrilton, Arkansas (15010-1041.23)
D. littoralis	Merlingen, Switzerland (15010-1001)
D. montana	Mount Hood National Forest, Oregon (15010-1021.19)

We used the strains listed in Table 1. All except the "white" strain of *D. virilis* and the *D. americana* Red Cloud strain were provided by the National Drosophila Species Resource Center, Bowling Green State University, Bowling Green, Ohio.

Because *D. virilis* is the only species in the group with stocks of *X*-linked mutations, this species was used in all crosses. *D. virilis* flies homozygous or hemizygous for *white* were crossed reciprocally to a second species; the F_1 females from each of these crosses were backcrossed separately to males from both parental species (in backcrosses to *D. virilis* we again used the *white* strain). These backcrosses yield two genotypes in males: *white* (carrying an *X*-linked segment from *virilis*), and *wild-type* (carrying an *X*-linked segment from the other species). Because recombination can occur in hybrid F_1 females, the *white* locus does not necessarily identify the species origin of the entire *X* chromosome. Instead, it marks the origin of an *X* chromosome segment: on average, the *white* marker remains associated with 100 map units of *virilis X* chromosome after one generation of backcrossing (CROW and KIMURA 1970, p. 95). However, because many of the species we used are either fixed or segregating for different *X* chromosome gene arrangements

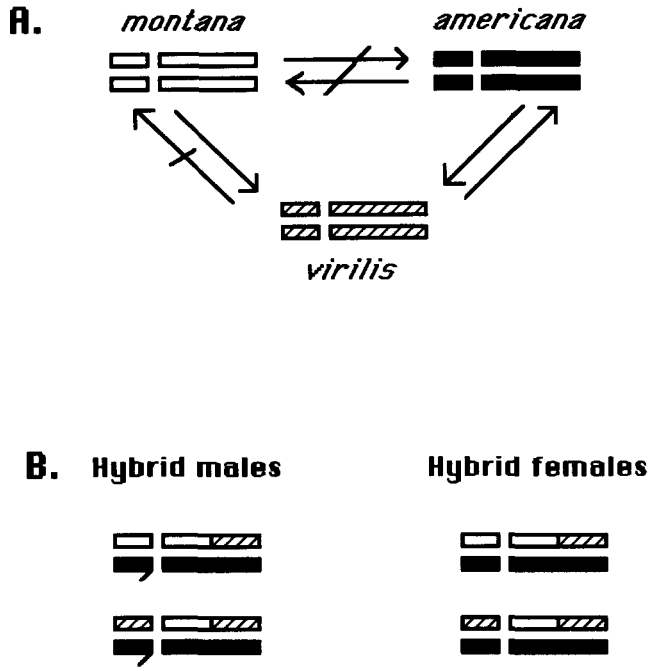

FIGURE 2.—"Three-way cross." A) *D. montana* and *D. americana* produce sterile hybrids of both sexes, barring direct study of the genetics of hybrid sterility. Each species, however, produces fertile hybrids when crossed to *D. virilis*. Arrows without bars represent successful crosses between species; arrows with bars represent crosses that produce all sterile or inviable offspring (see text for details). B) *D. virilis* can be used to pass *D. montana* chromosomes into a *D. americana* background, producing the male and female genotypes shown. On average, these genotypes differ only in the species origin of the *X* chromosome (the sex chromosomes are represented by short horizontal boxes; the autosomes are represented by the long horizontal boxes).

that reduce recombination (PATTERSON and STONE 1952, p. 447, Figure 69), the *white* marker will often mark more than 100 map units of the *X* chromosome.

Female offspring of the backcrosses to *D. virilis* also segregate for white and wild-type eyes; but females from the reciprocal backcross all have wild-type eyes because they are a mixture of *wild-type* homozygotes and *white* heterozygotes. These classes can be distinguished by progeny testing.

Three-way cross: Figure 2 shows the crossing relationships among *virilis*, *americana*, and *montana*. Both reciprocal crosses between *americana* and *montana* produce sterile male and female F_1 hybrids. All other crosses among the three species produce at least some fertile offspring of both sexes, except for the cross of *montana* females to *virilis* males, which yields completely sterile offspring. Because *virilis* can cross with both *americana* and *montana*, we can use it to transfer genetic material between them.

D. virilis white females were crossed to *D. montana* males, and the resulting F_1 females were crossed to *americana* males. The latter cross yields two male and two female genotypes. Within a sex, each genotype differs on average only in the species origin of the *X* chromosome (Figure 2). This allows us to compare the fertility of males carrying a *montana X* chromosome in a predominantly *americana* genome (50% of the autosomes and the *Y* from that species) with the fertility of males differing only by their possession of a *virilis X*. Figure 2 shows that these two classes should be *equally* fertile if the sterility of *americana-montana* hybrid males results from incompatibilities between the autosomes. If sterility involves the *X* chromosome, however, the former genotype should be more sterile than the latter. The effect of the *X* on *americana-montana* hybrid female sterility can be tested in a similar way (Figure 2).

As in our previous studies (COYNE 1984, 1985a; ORR 1987, 1988), male fertility was scored by the presence of motile sperm. These crosses were maintained at 18°. On the day of hatching, males were placed at 24° and retained until scoring for sperm motility (both *white* and *wild-type* males were stored together to eliminate vial effects). For crosses within the *virilis* phylad, males were scored on the 10th day after eclosion. Those hybrids involving members of the *montana* phylad (or the pure species within this phylad) were scored after fifteen days, as full sperm motility developed more slowly in this phylad.

Testes were removed from males of a given age, crushed in Ringer's solution, and examined under a compound microscope. Those males with one or more motile sperm were scored as "fertile," while those with immotile or no sperm as "sterile." In a few crosses, we subclassified "sterile" males into those having mature, differentiated sperm and those lacking any mature, differentiated sperm.

As in our previous studies (ORR 1987, 1988), female fertility was scored as the ability to produce larvae when inseminated. All experimental hybrid females were mass-mated with *virilis white* males for seven days at 18°. Each female was then placed in an individual vial with at least one *white* male. In intraphylad crosses, these vials were scored for larvae at the end of an additional 7 days. In interphylad crosses, vials were examined for the presence of larvae 14 days after females were isolated.

A female producing one or more larvae was considered fertile. If a female produced no larvae, her reproductive tract was removed in Ringer's solution and inspected for sperm under phase contrast. If sperm were present, the female was considered mated and sterile; if none were present, unmated. Vials that housed "sterile" females were retained for an additional 7 days; if larvae appeared the female was reclassified as fertile.

Backcrosses to species other than *D. virilis* yield two phenotypically indistinguishable female genotypes (w/w^+ and w^+/w^+). To estimate the relative fertilities of these genotypes, we scored the number of females producing white offspring when crossed to *virilis white* males. Only vials producing more than six progeny were scored, giving under Mendelian segregation a probability of greater than 98% of correctly identifying a female's genotype. This measure obviously confounds female fertility with viability: if few backcross females produce white offspring, we cannot determine whether w/w^+ females are indeed sterile or whether they fail to appear in the backcross generation in the expected numbers.

RESULTS

Males and females of all pure species are highly fertile: more than 90% of males have motile sperm and 95% of females produce progeny (Table 2).

Hybrid male fertility: Table 3 gives the fertility of F_1 and backcross males, and includes statistical analysis of the differences between reciprocal F_1s and of the differences between the two *X* chromosome genotypes in each backcross. As PATTERSON and STONE (1952) reported, F_1 males in several of these crosses are sterile (*e.g.*, *virilis/texana* and *virilis/novamexicana*). Both of these hybridizations show asymmetric male sterility so that F_1 males with *virilis* mothers are semisterile, but

TABLE 2

Fertility of males and females in pure species

Species	Sex	Fertile	Total	Proportion fertile
virilis w	Male	207	211	0.98
	Female	81	81	1.00
virilis y, ap	Male	208	216	0.96
	Female	74	74	1.00
texana	Male	203	207	0.98
	Female	79	79	1.00
lummei	Male	154	156	0.99
	Female	68	69	0.96
americana	Male	167	185	0.90
	Female	78	80	0.98
novamexicana	Male	204	205	0.99
	Female	113	113	1.00
littoralis	Male	174	185	0.94
	Female	85	88	0.97

Criteria for fertility are given in the text.

TABLE 3

Fertility of hybrid F_1 and backcross males

Parental cross	*X* chromosome genotype	Fertile	Total	Proportion fertile	*G* value
VT	*w*	129	195	0.66	60.7**
TV	w^+	199	209	0.95	
(VT)V	*w*	142	150	0.95	54.9**
	w^+	103	167	0.62	
(VT)T	*w*	98	116	0.84	13.9**
	w^+	192	199	0.96	
VA	*w*	185	200	0.92	6.3*
AV	w^+	210	215	0.98	
(VA)V	*w*	212	220	0.96	1.4
	w^+	200	213	0.94	
(VA)A	*w*	141	154	0.92	1.0
	w^+	181	192	0.94	
VLu	*w*	154	162	0.95	2.5
LuV	w^+	198	202	0.98	
(VLu)V	*w*	196	197	0.99	6.8*
	w^+	206	215	0.95	
(VLu)Lu	*w*	202	208	0.98	0.7
	w^+	197	206	0.96	
VN	*w*	15	229	0.07	162.1**
NV	w^+	43	44	0.98	
(VN)V	*w*	206	209	0.99	201.6**
	w^+	108	144	0.43	
(VN)N	*w*	33	152	0.22	163.4**
	w^+	153	172	0.89	
VLi	*w*	23	144	0.16	
(VLi)V	*w*	40	74 (13)	0.54	54.8**
	w^+	0	74 (43)	0.00	

In this and Tables 4–6, species names are abbreviated as follows: V = *virilis*, A = *americana*, T = *texana*, N = *novamexicana*, Lu = *lummei*, Li = *littoralis*, M = *montana*. In crosses, the species abbreviation for females is presented first, then males [*e.g.*, VT = virilis ♀ × *texana* ♂; (VT)V = F_1♀ (*virilis* ♀ × *texana* ♂) × *virilis* ♂]. In the (VLi)V cross, the number in parentheses refers to the number of immotile males with *no* mature, differentiated sperm. All *G* statistics are from heterogeneity *G* tests.

* $P < 0.05$; ** $P < 0.001$.

males from the reciprocal cross are perfectly fertile.

The reciprocal backcrosses of *virilis/texana* and *virilis/novamexicana* F_1 females to the two parental species show a similar effect of the *X* chromosome: those backcross males with an *X* chromosome from the paternal species (and hence with an *X* conspecific with the *Y* and most of the autosomes) are fertile, while males carrying the heterospecific *X* are often sterile. There is also a small but significant effect of the *X* chromosome in one of the *virilis/lummei* backcrosses. In the *virilis/americana* hybridization, all F_1 and backcross male genotypes are highly fertile. As one might expect, then, backcross male sterility appears only in those hybridizations producing sterile F_1 males.

In all hybridizations within the *virilis* phylad (including those three cases showing significant *X*-effects), backcross males carrying the "right" *X* chromosome are as fertile or nearly as fertile as males from the pure species, despite the fact that they carry on average one-quarter (and up to one-half) of their autosomes from another species. *This demonstrates that, within the virilis phylad, the autosomes have little or no effect on hybrid male fertility when heterozygous.* It is obvious that if the autosomes do play a role in F_1 male sterility, they must do so when heterozygous; but we have no evidence for this from the backcrosses.

The *X* chromosome also has a large effect on fertility in our only inter-phylad hybridization (*virilis* × *littoralis*). In the single possible backcross involving these species, males lacking the appropriate *X* never have motile sperm. In this cross we subdivided the "nonmotile" class into those males with differentiated but immotile sperm, and those with no differentiated sperm (*i.e.*, no sperm or only immature sperm bundles). The comparison of these two categories within the "nonmotile" class also reveals an effect of the *X* that borders on significance: males with the "wrong" *X* are less likely to have fully formed sperm (G_1 = 3.71; $0.06 < P < 0.05$). In contrast to hybridizations within the *virilis* phylad, there is substantial background sterility in backcross males with the "right" *X* chromosome, so the autosomes may affect fertility. We plan to test this possibility.

We can test the effect of the *Y* chromosome on hybrid fertility by crossing males from the two reciprocal F_1s back to a single parental species. The male offspring of these two backcrosses have identical *X* chromosomes, cytoplasm, and autosomes, and differ only in the species origin of the *Y*. We made two sets of such crosses using *virilis/texana* and *virilis/novamexicana* hybrids (these are the two hybridizations that show substantial backcross male sterility; Table 3).

TABLE 4

Effect of the *Y* chromosome on sperm motility

Genotype	Fertile	Total	Proportion fertile	
X^VY^V	182	190	0.96	$G_1 = 21.12$ ($P < 0.001$)
X^VY^T	134	166	0.81	
X^VY^V	28	36	0.78	$G_1 = 0.63$ (NS)
X^VY^N	73	87	0.84	

In the *virilis/texana* hybridization, the presence of a heterospecific *Y* chromosome reduces fertility by about 15%, an effect that is significant but only half as large as that of the *X* chromosome in a similar backcross (Table 4). In the *virilis/novamexicana* hybridization, on the other hand, the *Y* chromosome has no effect on male fertility despite a very large effect of the *X* (Table 4). Thus, as in other *Drosophila* groups, the *Y* chromosome occasionally has a large effect on fertility. Its role in postzygotic isolation, however, is not as consistent as that of the *X* (COYNE and ORR 1989a).

Hybrid female fertility: With the exception of females from the interphylad cross of *virilis* females to *littoralis* males, F_1 females from all hybridizations are highly fertile (Table 5). Backcross females, on the other hand, are often sterile (Table 5). This pattern differs from that seen among males, who are sterile in the backcross generation only if F_1 males are sterile.

The *X* chromosome has a significant effect on the fertility of backcross females. We can compare this effect with that of the autosomes in our backcrosses to *virilis*. We can assess the effect of the autosomes in these crosses by comparing the fertility of the white backcross females with the fertility of pure *virilis* females (these females differ only in their autosomal genotype). Unfortunately, we are unable to compare the effect of the *X* with that of the autosomes in our backcrosses to non-*virilis* species: because we cannot phenotypically distinguish backcross females who are homozygous for the *X* from heterozygotes, we cannot compare the fertility of the homozygous females with pure species females. Thus, in these crosses, we cannot assess the effect of the autosomes on fertility, nor compare this effect with that of the *X*.

In every hybridization between species in the *virilis* phylad, the sterility of backcross females is completely attributable to the *X* chromosome (*virilis/texana*, *virilis/americana*, *virilis/lummei*, and *virilis/novamexicana*; Table 5). Females carrying both *X* chromosomes from the same species are always fertile despite having up to 50% of their autosomes from a foreign species. As in the males, the autosomes have no detectable effect on female fertility when heterozygous. The only exception to this pattern in the interphylad cross between *virilis* and *littoralis* (Table 5), where the effect of the *X* chromosome substitution is in the expected direction but not statistically significant. In this cross many females with both *X* chromosomes from the same species are sterile, so that the autosomes may be involved (see below).

TABLE 5

Fertility of hybrid F_1 and backcross females

Parental cross	*X* chromosome genotype	Fertile	Total	Proportion fertile	*G* or χ^2
VT	w/w^+	86	87	0.99	1.26
TV	w/w^+	109	113	0.97	
(VT)V	w/w	107	109	0.98	14.48**
	w/w^+	116	136	0.85	
(VT)T	w/w^+	105			0.01
	w^+/w^+	104			
VA	w/w^+	89	89	1.00	2.91
AV	w/w^+	90	93	0.97	
(VA)V	w/w	121	123	0.98	4.68*
	w/w^+	120	129	0.93	
(VA)A	w/w^+	63			6.84*
	w^+/w^+	37			
VLu	w/w^+	58	60	0.97	0.00
LuV	w/w^+	118	122	0.97	
(VLu)V	w/w	91	91	1.00	11.37**
	w/w^+	106	120	0.88	
(VLu)Lu	w/w^+	104			7.59*
	w^+/w^+	68			
VN	w/w^+	88	89	0.99	0.78
NV	w/w^+	69	69	1.00	
(VN)V	w/w	125	127	0.98	18.43**
	w/w^+	78	94	0.83	
(VN)N	w/w^+	89			0.01
	w^+/w^+	88			
VLi	w/w^+	14	16	0.88	
(VLi)V	w/w	44	53	0.83	0.68
	w/w^+	50	65	0.77	

All *G* and χ^2 statistics are heterogeneity statistics (SOKAL and ROHLF 1981), except in the backcross to non-*virilis* species (*e.g.*, (VT)T). Chi-square tests were used when some observed frequencies were zero as *G* cannot be calculated. In backcrosses to non-*virilis* species, female genotypes are recognizable only by progeny testing (see text). Therefore, we can report only the number of viable/fertile females of each genotype. For these crosses we test the observed numbers of w/w^+ and w^+/w^+ females against an expected 1:1 ratio.

* $P < 0.05$; ** $P < 0.001$.

In backcrosses of F_1 females to the non-*virilis* parental species, two out of four hybridizations show a significant *X*-effect on hybrid fertility or viability (*virilis/lummei*, *virilis/americana*; Table 5). Surprisingly, however, these effects are in the opposite direction to those observed in the reciprocal cross: females with heterospecific *X* chromosomes are *more* fertile (or viable) than those with homospecific *X* chromosomes, despite the fact that the *X* chromosomes from the latter genotype are more compatible with the autosomes. This implies a maternal effect on hybrid viability or fertility (see DISCUSSION).

TABLE 6

Dissection of *X*-effects in cross between *D. virilis* and *D. novamexicana*

Cross	Sex	Genotype	No. fertile	Total	Proportion fertile
(VN)V	Male	*y, ap*	192	201	0.96
		y, ap$^+$	60	150	0.40
		y$^+$, *ap*	113	121	0.93
		y$^+$, *ap*$^+$	182	200	0.41
	Female	*y, ap*	67	68	0.99
		y, ap$^+$	60	62	0.97
		y$^+$, *ap*	89	91	0.98
		y$^+$, *ap*$^+$	67	96	0.70
(VN)N	Male	*y, ap*	13	46	0.28
		y, ap$^+$	96	118	0.81
		y$^+$, *ap*	7	58	0.12
		y$^+$, *ap*$^+$	109	139	0.78

F_1 female hybrids between *D. virilis yellow, apricot* females and *D. novamexicana* males were backcrossed to either parental species and recombinant and nonrecombinant offspring scored for fertility. Female offspring were not scored in the backcross to *D. novamexicana* because all females produced in this cross are fertile.

Genetic dissection of *X*-effects: We can determine whether the *X* chromosome harbors more than one "hybrid sterility gene" by recombinational analysis using a doubly marked *X* chromosome from *D. virilis*. This analysis also reveals whether the effects of the two *X* chromosome segments differ between males and females, and hence whether their hybrid sterility is due to different genes in the two sexes. We studied recombination on the *X* in only the *virilis/novamexicana* hybridization, because this cross alone produces backcross males and females that are frequently sterile. Although these two species differ by a sizeable inversion on the *X* chromosome that prevents recovery of some recombinants (PATTERSON and STONE 1952), we were able to obtain a reasonable number of recombinants.

We crossed *yellow, apricot virilis* females to *D. novamexicana wild-type* males, and backcrossed the resulting F_1 females separately to *D. novamexicana* and to *D. virilis yellow, apricot* males. Male offspring from both backcrosses are of four genotypes: *y ap*; *y ap*$^+$; *y*$^+$ *ap*; and *y*$^+$ *ap*$^+$. Females were studied in only the backcross to *virilis*, because only this backcross produces any sterile hybrid females (Table 5).

Almost all male sterility in both backcrosses is due to the segment of the *X* chromosome that includes the *apricot* locus (Table 6). In the backcross to *virilis*, substitution of the wild-type allele of *apricot* halves the fertility of males, while the analogous substitution at *yellow* has no effect. Statistical analysis using the CATMOD procedure of SAS (SAS Institute, Inc.) shows that *apricot* is linked to a gene or genes with a significant effect on hybrid fertility (maximum likelihood $\chi^2 = 140.5$, 1 d.f., $P < 0.001$); loci linked to *yellow*, however, have no detectable effect on fertility ($\chi^2 = 0.46$, $P > 0.40$). Both segments have a significant effect on male fertility in the backcross to *novamexicana*, but the segment linked to *apricot* again has a larger effect ($\chi^2 = 87.7$, $P < 0.001$) than that linked to *yellow* ($\chi^2 = 4.2$, $P = 0.04$).

TABLE 7

Fertility of males and females from the pure species stocks used in the "three-way" cross and from the F_1 hybridizations of *D. virilis*, *D. americana* and *D. montana*

Males	Motile	Immotile	None
D. virilis white	97	2	1
D. americana Red Cloud	80	18	0
D. montana Mt. Hood	41	13	0
AM	0	0	2
MA	0	0	19
VA	23	19	0
Females	**Fertile**	**Sterile**	**Unmated**
D. virilis white	81	0	1
D. americana Red Cloud	47	1	6
D. montana Mt. Hood	77	8	11
AM	0	1	0
MA	0	25	0
VA	76	1	0

The backcross to *virilis* (Table 6) shows that both halves of the *X* significantly affect *female* fertility (*y* segment: $\chi_1^2 = 4.2$, $P = 0.04$; *ap* segment: $\chi^2 = 6.8$, $P = 0.009$). However, females are sterile only when they carry wild-type markers on *both* halves of the *novamexicana X* chromosome—each half by itself has no effect. This effect requires at least two loci interacting epistatically.

Obviously, different chromosome segments, and therefore different genes, are involved in male and female hybrid sterility. The *yellow* region has a significant effect on female but not on male fertility in the backcross to *virilis*, and epistasis for fertility is observed among females but not among males.

"Three-way" cross: Because the cross of *virilis/montana* F_1 females to *americana* males is difficult, we obtained only 88 "three-way" hybrids. These individuals potentially carry genes from three species.

Males and females of the three species are highly fertile (Table 7). As reported by THROCKMORTON (1982), F_1 males and females from the two reciprocal hybridizations of *americana* × *montana* are sterile (the very small sample of *americana* female × *montana* male hybrids reflects the great difficulty of this cross). On the other hand, F_1 males and females from the cross of *virilis* females to *americana* males are often fertile, confirming that the *virilis X* chromosome causes little sterility on an *americana* genetic background (Table 7).

Table 8 shows that in the "three-way" cross the *X* chromosome again has a large effect on the fertility of males and the fertility and/or viability of females (we cannot distinguish between these forms of isola-

TABLE 8

Fertility of males and females produced from "three-way" cross of F_1 ♀ (*virilis* ♀ × *montana* ♂) × *americana* ♂

Males					
Genotype		Motile	Immotile	None	
w		1	13	6	
w^+		0	1	16	
Females					
w offspring	No *w* offspring	Unscorable	Sterile	Unmated	Dead
19	0	4	9	6	13

A female was classified as "unscorable" if she produced fewer than six offspring, all of whom were wild type.

tion in females because we do not know if the phenotypically identical +/*w* and +/+ female genotypes appear in the expected 1:1 ratio). Males carrying a *montana X* chromosome on a largely *americana* genetic background almost never have mature sperm, and none have motile sperm. In contrast, males differing from these only by their possession of a *virilis X* chromosome usually have mature sperm, and one had weakly motile sperm. This difference between the *X* chromosome classes is highly significant ($\chi^2 = 15.69$, 2 d.f., $P = 0.0004$). Because even males who carry the "good" *virilis X* chromosome usually produce immotile sperm, the autosomes may also play a role in *americana/montana* hybrid male sterility.

The *X* also has a large effect on hybrid *female* fertility/viability (χ^2 for observed versus 1:1 expected = 9.783, 1 d.f., $P < 0.0018$; this test is conservative because we assume that all four "unscorable" females in Table 8 were +/+). Of the 19 scorable females, all had some white-eyed progeny, demonstrating that they carried the *virilis* and not the *montana X*. The *montana X* chromosome obviously has a large effect on hybrid sterility or inviability when placed on an *americana* genetic background.

DISCUSSION

Postzygotic isolation is an important aspect of speciation in *Drosophila*, evolving as rapidly as mating discrimination when two populations become geographically isolated (COYNE and ORR 1989b). Two patterns characterize the evolution of postzygotic isolation in *Drosophila*: HALDANE's rule and the large effect of the *X* chromosome. As PATTERSON and STONE (1952) and THROCKMORTON (1982) note, the *D. virilis* group obeys HALDANE's rule: when only one hybrid sex is sterile or inviable, it is almost invariably the males. The *D. virilis* group also obeys our second rule, for the genes having the largest effect on both hybrid male and female sterility are always *X*-linked. We report four new cases of the large *X*-effect among hybrid males and four new cases among hybrid females; this is a substantial addition to the nine hybridizations that have been previously genetically analyzed in *Drosophila* (COYNE and ORR 1989a).

It is of course possible that these hybridizations do not represent independent cases of the evolution of *X*-linked sterility. To establish this would require using *X*-linked markers in hybridizations among all possible species pairs, a task that is presently impossible because markers are available only in *D. virilis*. Fortunately, however, we can infer from the group's phylogeny that several of the observed *X*-effects are probably evolutionarily independent events. Considering males, it is likely that the appearance of *virilis/texana* and *virilis/novamexicana* sterility represent two independent evolutionary events because *virilis/americana* and *virilis/lummei* F_1 and backcross hybrids are fertile (see phylogeny in Figure 1). Moreover, because the *X* has a *complete* effect on hybrid sterility only in the crosses involving *virilis* and *littoralis*, this effect probably also represents an independent evolutionary event.

This study greatly increases the number of cases of hybrid *female* sterility or inviability that have been genetically analyzed [see COYNE and ORR (1989a) for references]. We find that the *X* chromosome affects the fertility of these females as consistently as it does that of males. We first consider the backcrosses to *D. virilis*. The *X* has a significant effect on female sterility in all four of our intraphylad hybridizations: *virilis/lummei*, *virilis/texana*, *virilis/novamexicana*, and *virilis/americana*. (The segment of chromosome four fused to the *X* in *D. americana* is probably not responsible for the effect of the *X* in this cross: the sterility genes must be linked to the *white* locus (l-105) which is near the middle of the *X* and thus quite distant from the fused fourth segment.) Moreover, in all four intraphylad hybridizations, females carrying conspecific *X* chromosomes are as fertile as females of pure species, despite the fact that on average 25% of their autosomes are from a foreign species. Just as for the intraphylad hybrid males, then, the heterozygous foreign autosomes alone have little or no effect on female fertility. A foreign *X* chromosome, however, does cause sterility when heterozygous.

Because hybrid sterility occurs *only* when backcross males and females carry an inappropriate *X* chromosome, it must result largely from the action of *X*-linked alleles. The *X* must of course interact with some genetic element from the other species to cause sterility, because it does not do so on its normal genetic background. But if sterility results from disrupting a network of interactions between *X*-linked and autosomal genes, and two species have diverged far more at *X*-linked than autosomal loci, then substitution of an inappropriate *X* will cause sterility far more than substitution of an inappropriate autosome. Therefore, our failure to observe any significant autosomal effect

does not imply a complete absence of autosomal "sterility genes"—only that they are fewer in number and smaller in effect. In addition, there may be cytoplasmic or *Y*-linked sterility factors that interact with those on the *X*, such as those seen in our cross between *virilis* and *texana*.

In the single *inter*phylad cross (*virilis* × *littoralis*), on the other hand, the autosomes may affect hybrid fertility, for here females are often sterile when they carry the "correct" *X* chromosomes. While this is consistent with an autosomal effect, we cannot rule out the possibility of a cytoplasmic effect, and plan further crosses to test this possibility.

The *virilis/littoralis* cross is the only hybridization that shows no significant effect of the *X* chromosome on fertility/inviability under our normal protocol of scoring female fertility at week 3. However, the difficulty of this hybridization produces very small samples, and there is a small and non-significant (6%) fertility difference in the expected direction between the *X* chromosome genotypes. Moreover, when fertility is scored at week 2, the *X* chromosome does have a large and significant effect on "fertility" (data not shown, $G = 5.24$, $P < 0.05$); no other hybridization yields different results when fertility is scored at week 2 *vs.* 3. Because this *X*-effect disappears when the vials are reexamined at week 3, it may involve effects on hybrid development time.

Backcrosses to the non-*virilis* species are less informative because the two *X* chromosome genotypes are distinguishable only by progeny testing. As a result, we cannot separate sterility and inviability effects. Because we do not know the ratio of w/w^+ to w^+/w^+ females actually appearing in a backcross (there may be *X*-effects on viability), we do not know how many females of each genotype are actually fertile. In these cases, we can test for an *X*-effect by comparing the number of females producing some white versus all wild-type offspring. If the *X* chromosome has no effect on hybrid female fertility and viability, we expect these broods to appear in a 1:1 ratio. We are also unable to compare the effect of the *X* with that of the autosomes as we could in the backcrosses to *white* males, for we do not know the proportion of w^+/w^+ females that are actually fertile (as these females differ from pure species females only by having about 25% of their autosomes from a foreign species, information on their fertility would allow a test of the effect of the autosomes on fertility).

It is worth noting, however, that two hybridizations (*virilis/lummei* and *virilis/americana*) show a significant effect of the *X* chromosome on female fertility or viability. This effect is, however, in an unexpected direction—females heterospecific for the *X* chromosomes are *more* fertile/viable than homozygotes despite the greater compatibility between *X*s and autosomes in the latter genotype. This suggests that there are maternal effects on female fertility/viability that involve an interaction with the *X* chromosome. Perhaps the *lummei* and *americana* *X*s are compatible with cytoplasm derived from "pure" species females (as the high F_1 fertility shows), but incompatible with cytoplasm derived from hybrid F_1 females. Alternatively, backcross females homozygous for *lummei* or *americana* *X*s may produce inviable offspring because of a maternal effect, and thus appear sterile. MITROFANOV and SIDOROVA (1981) describe a maternal effect on *virilis/lummei* hybrid female viability that is generally consistent with our results [these workers, however, misidentified *lummei* as "*littoralis*" (THROCKMORTON, 1982)].

Our results also show that the large effect of the *X* on postzygotic isolation may result from the action of a few genes. We find, for instance, that white males and females produced in the backcrosses to *virilis* are just as fertile as pure species, despite the fact that they must often carry recombinant *X* chromosomes. This suggests that hybrid sterility results from *X*-linked genes that are fairly tightly linked to the *white* locus. If many sterility genes were scattered along the *americana*, *texana*, *novamexicana*, and *lummei* *X* chromosomes, these genes would often become linked to the *white* allele through recombination, and white individuals would sometimes be sterile. Also, in our only recombination study (*virilis/novamexicana*), we found that the genes causing hybrid male sterility are restricted to one end of the *X*. Unless there are "hot spots" for the accumulation of hybrid sterility genes, this again implies the involvement of rather few genes.

These are not the only cases in which postzygotic isolation apparently results from a small number of genes. ORR (1988, and in preparation) showed that the *X*-linked genes causing male sterility in *D. pseudoobscura* Bogota/USA hybrids are restricted to less than 20% of the chromosome, implying that one or a few loci are involved. Sterility in other hybridizations, however, apparently involves more *X*-linked loci. NAVEIRA and FONTDEVILA (1986) found that even small segments of the *D. serido* *X* chromosome cause male sterility when introgressed into the sibling species *D. buzzatti*. This implies the existence of many sterility genes. *D. pseudoobscura* and *D. persimilis* have diverged at a minimum of four or five *X*-linked loci affecting hybrid male fertility (ORR 1987). Similarly, *D. simulans* differs from *D. mauritiana* and *D. sechellia* at a minimum of three *X*-linked loci affecting hybrid male fertility (J. A. COYNE and B. CHARLESWORTH, unpublished data).

Our results support the notion that these "speciation genes" accumulate on the *X* chromosome as a result of natural selection. Two types of favorable mutations should accumulate faster on the *X* than on the auto-

somes. As CHARLESWORTH, COYNE and BARTON (1987) emphasize, advantageous but partially recessive mutations are at best neutral when they first appear on an autosome, for their advantageous effects are largely masked in heterozygous state. Such mutations can, however, be immediately selected in males if they are *X*-linked. COYNE and ORR (1989a) further show that partially recessive mutations affecting hybrid males will accumulate faster than those affecting females if there is some correlation between the sex in which a mutation was originally favored and the hybrid sex it later afflicts. This theory can therefore explain both HALDANE's rule and the large *X*-effect, although it requires that most favorable mutations are very recessive.

Similarly, mutations that produce favorable homozygotes but inferior heterozygotes are substituted much faster when *X*-linked than when autosomal. In such an underdominant system, a new autosomal mutation is immediately eliminated because it occurs exclusively in heterozygotes. If *X*-linked, however, it can be fixed if its favorable effects in hemizygous males outweigh its deleterious effects in heterozygous females. As a result, such mutations will accumulate much faster on the *X* chromosome than on an autosome. The difference in substitution rates depends, of course, on the heterozygous disadvantage of the mutant and the effective population size (CHARLESWORTH, COYNE and BARTON 1987).

Although CHARLESWORTH, COYNE and BARTON (1987) considered only the fixation of underdominant chromosome rearrangements, their model also applies to mutations at individual loci. Mutations producing favored homozygotes but disadvantageous heterozygotes could, for example, be common at loci producing multimeric proteins (the heterodimers could be less efficient than either homodimer). The "pleiotropy theory" discussed by COYNE and ORR (1989a) can easily be extended to underdominant mutations: those mutations with a larger effect in males than in females will obviously be fixed much more frequently than those with the opposite effects. Therefore, if there is a correlation between the sex that a mutation originally affects and the hybrid sex it ultimately afflicts (as seems reasonable), then hybrid males will be affected more than hybrid females. The fixation of underdominant mutations may then also explain HALDANE's rule.

The "underdominant" and "recessivity" theories make several predictions that can be tested with our data from the *virilis* group. First, if advantageous mutations accumulate faster on the *X* chromosome than on autosomes, and such mutations ultimately cause postzygotic isolation, the *X* chromosome might affect the fertility or viability of hybrids before the accumulation of many such genes on the autosomes. Hybrid sterility or inviability between two closely related species would then be due almost entirely to the *X* chromosome, while that of hybrids between more distantly related species would be due to all of the chromosomes. Our data support this prediction: in *intra*phylad crosses, only the *X* chromosome has a significant effect on male and female fertility, while in *inter*phylad crosses even males and females with the appropriate set of *X* chromosomes may be sterile (implying that the autosomes also play a role).

The second prediction follows from COYNE and ORR's (1989a) "pleiotropy theory": the effect of the *X* chromosome relative to an autosome of similar size will be greater for hybrid males than for females, but this ratio will exceed one for both sexes. We have already shown that among male *and female* hybrids, the *X* chromosome always has a larger effect on sterility than all the autosomes combined. Obviously, then, the "X/A ratio" exceeds one for both sexes. We can also show that the ratio of *X* to autosomal effects is greater among male than among female hybrids: while the autosomes have no perceptible effect in any intraphylad cross, the *X* has a larger effect in males than in females (Tables 3 and 5). The effect of the *X* relative to autosomes must therefore be greater for hybrid males than for females, supporting the "pleiotropy" explanation of HALDANE's rule. As we note below, the most widely accepted explanation of HALDANE's rule—that of MULLER—cannot explain why the *X* plays such a large role in hybrid *female* sterility and inviability.

These recent theories do, however, require one questionable assumption: that the favorable mutations capable of causing postzygotic isolation were originally partially recessive or underdominant. We know of no independent evidence for this assumption. Furthermore, it is not clear why characters *other* than hybrid sterility and inviability, such as morphological differences between related species, do not show a large *X* chromosome effect upon genetic analysis (CHARLESWORTH, COYNE and BARTON 1987). Until we can gather more evidence supporting the assumption of recessivity or underdominance, our explanations of HALDANE's rule and the large *X*-effect must remain tentative.

Despite this weakness of the theory, it must be noted that the genetics of sterility and inviability in hybrid *females* rule out the only alternative explanation of HALDANE's rule and the large *X*-effect: MULLER's "hemizygosity hypothesis." MULLER (1940, 1942) contended that preferential sterility and inviability of the heterogametic sex results from their possession of a single *X* chromosome. Heterogametic hybrids therefore suffer from a greater *X*/autosomal "imbalance" than homogametic hybrids because the former lack an *X* chromosome conspecific with a haploid set of

autosomes. MULLER made this argument more genetically explicit by suggesting that many alleles causing postzygotic isolation are recessive. In this case, one should observe not only HALDANE's rule (the heterobut not the homogametic sex shows the full effect of *X*-linked recessives and thus would be sterile or inviable more often), but also a large effect of the *X* chromosome. Thus MULLER's argument does *not* posit that the *X* chromosome accumulates "hybrid sterility/inviability" alleles more rapidly than autosomes, but merely that such alleles are more frequently *expressed* when on the *X*.

Three lines of evidence, however, falsify MULLER's explanation. First, hybrid *females* with an *X*/autosomal imbalance as severe as that of sterile or inviable F_1 hybrid males are perfectly viable and fertile [see the evidence in COYNE and ORR (1989a) and *w*/*w* females in Table 5 of present paper]. Second, when hybrid females *are* sterile or inviable, the *X* chromosome still has the largest effect of any chromosome, despite the fact that in females it is not hemizygous but equivalent in dosage to an autosome (Table 5). Third, the *X* chromosome usually has a large effect on postzygotic isolation when *heterozygous*, not when homozygous (Table 5), which is the opposite of what MULLER's hypothesis predicts.

Three-way cross: This method allows us to determine the genetic basis of reproductive isolation in hybridizations that are traditionally unanalyzable, *i.e.*, those producing no viable, fertile F_1 hybrids. The only previous method allowing analysis of such hybridizations was MULLER and PONTECORVO's (1940) and PONTECORVO's (1943a, b) use of genetically marked triploids to investigate the fertility and viability of *D. melanogaster*/*D. simulans* "partial hybrids." Unfortunately, their method cannot be widely applied because it requires special genetic stocks available only in *D. melanogaster*.

Analysis of hybridizations producing completely sterile or inviable hybrids is useful for two reasons. First, if species pairs producing such hybrids are older than those yielding less postzygotic isolation (see below), we are able to study the genetic changes that complete speciation. This, of course, is normally impossible because the complete reproductive isolation itself bars genetic analysis (we should point out that three-way crosses could also be used to study the genetics of *pre*zygotic isolation between two species that never mate). Second, whether or not the age of a species pair is correlated with the sterility or inviability of their hybrids, our technique allows one to determine whether the genetics of reproductive isolation differ between completely and partially isolated species pairs (*e.g.*, does the *X* chromosome also have a large effect among completely isolated taxa?). The argument that the *X*-effect is an artifact of analyzing cases of HALDANE's rule (see introduction) obviously predicts that completely isolated species will not show such an effect because their isolation results from incompatibilities between autosomes.

Our analysis of *americana*/*montana* hybrid sterility reveals that the *X* does have a large effect on the fertility of both male and female hybrids in this traditionally unanalyzable cross. If the sterility of *americana*/*montana* hybrid males were due to incompatibilities between the autosomes, the two classes of males shown in Figure 2 should be equally fertile. They are not. Instead, males carrying a *montana X* chromosome on a largely *americana* background are far more sterile than control males carrying the *virilis X*. Similarly, the two classes of females shown in Figure 2 have identical autosomal genotypes and should be equally fertile if the bias hypothesis were true. Again, they are not: females carrying a *montana X* chromosome apparently *never* produce progeny, while control females carrying a *virilis X* chromosome are both fertile and viable. Obviously, the *X* chromosome has a large effect on the sterility or viability of male and female *americana*/*montana* hybrids. The large *X*-effect is not limited to cases of HALDANE's rule, and therefore does not result from an empirical bias.

There is another line of evidence that falsifies the bias hypothesis. COYNE and ORR (1989b) show that HALDANE's rule is the earliest stage of speciation in *Drosophila*; indeed, it is the *only* pattern of postzygotic isolation observed in hybridizations between closely related species. This pattern shows there are not two parallel pathways to speciation, one involving *X*-effects (producing analyzable cases of HALDANE's rule) and the other involving autosomal-autosomal incompatibilities (producing unanalyzable cases of completely sterile or inviable hybrids). Instead, "unanalyzable" crosses represent the later stages of a process that begins with the appearance of HALDANE's rule. HALDANE's rule—and the large *X*-effects that underlie it—represent the beginnings of speciation in *Drosophila*.

In conclusion, by greatly expanding the number of genetic studies of postzygotic isolation, we strengthen the evidence that the genes playing the largest role in hybrid sterility and inviability are *X*-linked. Such *X*-effects are to be found in almost every genetic analysis of postzygotic isolation. We also provide two lines of evidence supporting the theory that postzygotic isolation results from the accumulation of favorable partially recessive or underdominant alleles by natural selection. Furthermore, in the only hybridization yet subjected to recombination analysis, we show that male and female sterility result from the action of *different X*-linked loci. Finally, we introduce a method that allows genetic analysis of species pairs that are completely reproductively isolated, *e.g.*, that produce all sterile or inviable hybrids. Using this method, we

show that the *X* chromosome has a large effect on postzygotic isolation in such species pairs, as well as in those obeying HALDANE's rule.

This work was supported by training grant GM 07197 from the National Institute of General and Medical Sciences of the National Institutes of Health to the University of Chicago, and by National Institutes of Health grant GM 38462 to J.A.C. We thank our three anonymous reviewers, J. GILLESPIE, S. JONES and C. LANGLEY, for their helpful comments. We thank C. LANGLEY for pointing out that the underdominance model discussed above applies at the level of a single gene.

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Communicating editor: M. TURELLI