Analysis of incipient reproductive isolation within a species of *Drosophila*

(subspecies/hybrid sterility/chromosomal analysis)

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ABSTRACT *Drosophila willistoni* and its subspecies *quechua* are morphologically indistinguishable, but differ in relative frequencies of certain allozyme genes. The cross *quechua* ♀ × *willistoni* ♂ produces nearly or completely sterile males, while the reciprocal cross gives fertile males. Hybrid females are fertile. Analysis of the sterility with the aid of chromosomes marked with mutant genes shows that the second and X-chromosomes have major and third chromosomes minor effects. Backcross males of the same chromosomal constitution may be either fertile or sterile; the threshold effect may be due to environmental variations or to gene variants present in the strains crossed. Only a trace of ethological isolation between the subspecies is present. In contrast to a narrowly localized and geographically isolated subspecies *bogotana* of *D. pseudoobscura*, the subspecies *quechua* of *D. willistoni* is fairly widespread, although its geographic area is not accurately known. Neither with *bogotana* nor with *quechua* are there valid reasons to suppose that these subspecies are of very recent origin.

Species in sexually reproducing and outbreeding organisms are arrays of Mendelian populations reproductively isolated from other population arrays. Reproductive isolation is thus an earmark of species distinction. However, the process of speciation, of genetic divergence and advent of reproductive isolation, is usually, though not always, a gradual one. Beginnings of reproductive isolation, the genetic building blocks from which isolating mechanisms are compounded, should be found within species. Materials suitable for genetic analysis of early stages of speciation are rare. One such material is *Drosophila willistoni* and its subspecies *quechua*, dealt with in the present article. Another is *Drosophila pseudoobscura* and its subspecies *bogotana*, reported upon elsewhere (1–4). The situations found in these two species are very similar in some but different in other respects. Their comparison is instructive.

MATERIALS AND METHODS

Drosophila willistoni Sturtevant is widespread and common in the tropics and subtropics of the Western Hemisphere, from southern Florida and Mexico to La Plata in Argentina. A population sample taken by Prof. C. Pavan in 1954 near Lima, Peru, proved to give sterile male hybrids with strains of the same species from Brazil. The cross Lima ♀ × Brazil ♂ gives completely sterile F₁ hybrid males, while the reciprocal cross gives fertile males. Female hybrids are fertile (5). Another sample of 38 inseminated females was obtained near Lima by Prof. D. Brncic in 1972. Strains derived from this sample behaved in crosses like those of the older sample. They were combined together to form two mixed stock cultures. Flies from these cultures served as materials for the

present study, and since the behavior of the two cultures was similar the data were pooled. Because of its behavior in crosses, and frequency differences of certain allozyme alleles studied by electrophoresis, the Lima population has been described as *Drosophila willistoni* subspecies *quechua* Ayala (6). Strains with dominant mutant markers Emarginate (Em), Star (S), and Delta (Δ) in the second and third chromosomes have been available for many years (7). These mutants were obtained in strains of Brazilian origin.

RESULTS

Geographic distribution and crossability

Spassky and the present writer made crosses of Lima strains of the subspecies *quechua* to a variety of strains from other localities (5). Sterile F₁ hybrid males were produced in crosses of *quechua* females to males from the following states and territories of Brazil: Acre, Rondonia, Amazonas, Roraima, Para, Maranhão, Goias, Parana, and Rio Grande Sul. Strains from Bucaramanga, Colombia, and from Simla, Trinidad, also gave sterile male hybrids. In contrast, fertile hybrids were obtained in crosses of *quechua* females to males from Mexico, Guatemala, Honduras, and Ecuador. The results of de Toledo (8) and Winge (9) are more extensive but not entirely consistent. Females of *quechua* produced fertile hybrid males not only with males from Florida, Cuba, Guatemala, and Ecuador, but also with several strains from central and southern Brazil. Florida females gave sterile hybrid sons with 23 out of 25 strains from various parts of Brazil, but fertile sons with Cuba, Guatemala, Ecuador, and Lima. Guatemala and Ecuador females give fertile hybrids with most strains from Brazil in the experiments of de Toledo (8) but not in those of Winge (9). The last-named author proposes to distinguish three "races" of *Drosophila willistoni*. The northern race inhabits Central America, West Indies (except Trinidad), and southern Florida. The southern race lives in Brazil, Argentina, Colombia, and Trinidad. A "transitional" race is found in Peru (Lima) and Ecuador. If this is confirmed, the name *quechua* would apply to the transitional race.

Further data are obviously needed to clarify the distribution areas of the populations giving sterile and fertile hybrids when crossed. It appears that there are various degrees of hybrid sterility between populations of *Drosophila willistoni*. This contrasts with a simpler situation in *Drosophila pseudoobscura* (2, 3). The subspecies *bogotana* which lives in the high Andes near Bogota, produces sterile male hybrids when used as a female parent with males from any other

population of the species. These latter are fully fertile *inter se*.

Lack of differences in the gene arrangements

The *quechua* population of Lima, and probably of other coastal valleys in western Peru, is isolated geographically from the main body of the species east of the Andes in the basin of the Amazon. The rampart of the Andes is uninhabitable for *D. willistoni*. Now, the Amazonian populations are chromosomally highly polymorphic, as are those of Colombia west of the Andes (10, 11). The mean numbers of heterozygous inversions per individual east of the Andes in Peru is between 6 and 6.5, and in eastern Colombia between 5 and 5.5. The inversion polymorphism at least partly persists in laboratory strains. The *quechua* stocks were examined cytologically, and proved to be largely homokaryotypic, although three inversions were found in occasional individuals (II L-E, II L-F, III-J). Low frequencies of inversion heterozygosis are found also in western Ecuador (Santo Domingo de los Colorados, Pichilingue), west of the Andes in Colombia, and in the West Indies (10, 11).

Lima stocks were outcrossed to strains of *D. willistoni* from Belem (Brazil) and from St. Kitts (West Indies), which are known to be virtually free of inversion polymorphisms. If the gene arrangements in Lima chromosomes were different from those in Belem and St. Kitts, the F_1 larvae would display inversion heterozygosis. No heterozygous inversions were found, except those few which came from the Lima parents. The subspecies *quechua* has no fixed differences from *willistoni* in the chromosome structure. The subspecific divergence can occur without alteration of the gene arrangements in the chromosomes. This is in no way surprising, since morphologically distinct and reproductively isolated species of Drosophila can also be homosequential, i.e., identical in gene arrangements (12, 13).

Genetic analysis of hybrid sterility

Reciprocal crosses of Lima (*quechua*) strains were made to Emarginate Delta (EM Δ) and Star Delta (S Δ) lines. The hybrid males were tested for fertility in mass cultures. Sons of Lima fathers were fertile. The reciprocal cross produced almost entirely sterile sons, although in some cultures isolated larvae and adults did appear. The sterility of the male progeny of Lima ♀ × Em Δ ♂ and Lima ♀ × S Δ ♂ is nearly, though not quite, complete.

Em Δ or S Δ sons from the crosses Em Δ ♀ × Lima ♂ or S Δ ♀ × Lima ♂ were selected and crossed to Lima females. Four classes appear in the progeny: wild, Delta, Emarginate (or Star), and Emarginate Delta (or Star Delta). All males have their X- and Y-chromosomes and one set of autosomes derived from Lima (*quechua*) parent or grandparent. Wild-type males have the other set of autosomes also of Lima origin, and thus are genetically identical with pure Lima. Delta males have one third chromosome from the marker stock, Emarginate or Star one second chromosome, and Emarginate Delta or Star Delta one second and one third chromosomes with markers (of Brazilian origin). Emarginate Delta and Star Delta males are similar in chromosomal constitution to F_1 hybrids from the cross Lima ♀ × Star Delta ♂, except that the male progeny of the latter cross have Y-chromosomes of Brazilian origin.

Males of the four classes were individually tested for fertility or sterility. Single males were placed with five to six virgin females of Lima or other *willistoni* strains. The ap-

Table 1. Incidence of sterility among males of different chromosomal constitution

Gene markers	Fertile	Sterile	Dead	Percent sterile
Wild	108	5	7	4.4
Delta	112	4	9	3.4
Em or S	81	35	5	30.2
Em or S Delta	71	59	5	45.4

pearance of larvae indicated that the male was fertile. Where no larvae appeared after about 10 days, the flies were etherized and inspected to see whether the male as well as the females were alive. If they were, the male was recorded as sterile. The results are shown in Table 1.

Wild and Delta males are almost all fertile. The presence of one non-Lima third chromosome on an otherwise *quechua* background does not cause sterility. One second chromosome, with Emarginate or with Star, gives about 30% sterile males, and one second plus one third chromosome about 45% sterility. It should be noted that the sterility of the Em Δ class is significantly more frequent than that of the Em class (chi-square 5.98, probability for 1 degree of freedom between 0.02 and 0.01). It appears that, although a single foreign third chromosome does not by itself produce sterility, it increases the frequency of sterility in combination with the second chromosome.

A notable aspect of the above results is the following. All males of each class with the same mutant markers, e.g., Em or Em Δ, have the same sets of *quechua* and non-*quechua* chromosomes. And yet only 30 or 45% of these males are sterile, while the rest are fertile. Why should there be such variation among individuals of the same chromosomal constitution? The variation is the same among males showing Em or S mutants as markers. The sterility-fertility is apparently a threshold phenomenon. Either environmental fluctuations or gene modifiers present in the strains used must be responsible for crossing or noncrossing of the threshold.

Cytology of sterile males

Testes of males from crosses giving nearly complete (F_1) or partial (backcrosses) sterility were examined in squash preparations under phase contrast microscope. The spermatogenesis is ostensibly normal, except that binucleated spermatocytes are more frequent than they are in nonhybrid males. In favorable cells three bivalents can be seen at the first meiotic division. Some males do and others do not have motile spermatozoa in the seminal vesicles. Presence of motile spermatozoa does not necessarily make a male fertile.

Ethological isolation

The frequencies of matings were recorded in mixtures of *quechua* and *willistoni* flies in observation chambers (14). Lima strains of *quechua* and Arima (Trinidad) and Mirassol (São Paulo, Brazil) of *willistoni* were used. Since the subspecies are indistinguishable in appearance, the tips of the wings of one of them were lightly clipped; the clipping was done alternately in *quechua* and *willistoni* flies in successive trials. Equal numbers (12) of males and of females of both kinds were introduced without etherization in an observation chamber (i.e., 48 flies total), and the matings which occurred during 2–3 hr after the introduction were observed. Most matings occur within the first half hour or less of obser-

Table 2.   Numbers of matings observed between *quechua* (Lima) and *willistoni* (Mirassol or Arima) flies

Strains	*quechua* ♀ *quechua* ♂	*quechua* ♀ *willistoni* ♂	*willistoni* ♀ *quechua* ♂	*willistoni* ♀ *willistoni* ♂	Isolation index
Lima + Mirassol	91	45	72	68	+0.152 ± 0.059
Lima + Arima	49	27	30	54	+0.287 ± 0.076
Total	140	72	102	122	+0.202 ± 0.047

vation; nevertheless, only rarely do all the females mate during the total observation time. The recorded numbers of the four classes of matings are shown in Table 2, together with Levene's isolation indices (15).

It can be seen that a very weak, but statistically assured, preference for homogamic matings exists between *quechua* and both strains of *willistoni* with which it has been tested. Nearly equal numbers of *quechua* and *willistoni* females (212 and 224) have been observed mating, but it appears that *quechua* females are somewhat reluctant to mate with *willistoni* Mirassol males, while *willistoni* females accept Lima and Mirassol males equally.

DISCUSSION

Though belonging to the same subgenus (*Sophophora*), *Drosophila willistoni* and *D. pseudoobscura* are only distantly related. Their gene arrangements are so radically altered that the homology of the chromosomal limbs cannot be recognized by the disk patterns in their salivary gland chromosomes. Nevertheless, both species have given rise to subspecies (*quechua* and *bogotana*, respectively) with remarkably similar properties. In both species one of the crosses (*quechua* ♀ × *willistoni* ♂ and *bogotana* ♀ × *pseudoobscura* ♂) gives completely or nearly sterile sons in the F_1 generation; the reciprocal crosses give fully fertile hybrids; hybrid females are fertile. Also in both species there is very little or no ethological isolation between the subspecies. Estimates of the amount of the genetic divergence have been obtained by examination of allozymes detected by electrophoresis (4, 16). The genetic distance is close to 0.20, which suggests that about 20% of the gene loci in the subspecies and the nominate forms differ by one or more codon substitutions. This compares with the average genetic distance of only 0.03 between local populations within a species and subspecies, 0.23 between semispecies, 0.58 between sibling species, and 1.06 between related nonsibling species (16). The incipient reproductive isolation, the partial hybrid sterility of the subspecies hybrids, has not arisen without genetic differentiation of the subspecies populations, as some authors mistakenly supposed. The genetic distance between the subspecies is about intermediate between the average distance separating fully compatible local populations and that separating the semispecies which are isolated enough to be capable of sympatric coexistence.

Genetic analysis of the male sterility in the subspecies hybrids shows that most or all chromosomes are involved. This is as true in *willistoni* as it is in *pseudoobscura* (2, 3). Probably there is more than a single "sterility gene" per chromosome, but very laborious experiments would be necessary to establish this conclusively. Furthermore, in both species there is an interesting, if somewhat puzzling, difference between the sterilities of the F_1 and the backcross males. The F_1 males are absolutely sterile (or nearly so in *willistoni*). Backcross males of identical chromosomal constitution may be sterile, or semisterile, or fertile (production of any progeny placed a male in the "fertile" column in Table 1). These

fertility-sterility variations may be due to maternal effects (3), or to environmental fluctuations, or finally to genetic modifiers present in the strains crossed. Anyway, the same environmental fluctuations and modifiers have little or no influence on the F_1 males. Crossing-over between the chromosomes of the two subspecies cannot be the cause, since in our experiments the backcross hybrids are the progeny of hybrid males rather than females. The sterility of the F_1 and backcross males is being studied further.

In only one respect are the conditions found in *D. willistoni* and *D. pseudoobscura* contrasting. The subspecies *bogotana* occupies a small territory in the high Andes, geographically far disjunct from the rest of the species *pseudoobscura*. It has been alleged that *bogotana* arose from ancestors introduced by man a quarter of a century ago (2), although new evidence makes this highly unlikely (3). The subspecies *quechua* of *D. willistoni* inhabits a much greater area, and populations transitional between it and *willistoni willistoni* may be found (9). To suggest that *quechua* is only a quarter of a century old would be farfetched in the extreme, even though the genetic situation of *quechua* resembles very much that of *bogotana*. It is, of course, possible that *quechua* arose first in a small territory and subsequently spread. What is notable is that hybrid sterility, a postmating isolating mechanism, develops before premating isolating mechanisms, such as ethological isolation. This is in accord with the hypothesis that postmating and premating isolating mechanisms arise in different ways (17, 18). Postmating mechanisms, such as hybrid inviability and sterility, are byproducts of genetic divergence; premating ones are contrived by natural selection to mitigate or eliminate the losses of fitness which result from hybridization of genetically divergent and differentially adapted forms.

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