Cytogenetic study of three closely related species of Hawaiian *Drosophila*

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Three allopatric species from the Hawaiian islands, *Drosophila bostrycha* (Molokai), *D. affinidisjuncta* (West Maui), and *D. disjuncta* (East Maui), are extremely similar in morphology but differ in metaphase chromosomes by the amount and distribution of heterochromatin. Their polytene chromosomes are virtually homosequential with only slight differences at the tip of the microchromosome. Each is polymorphic for one or more inversions, especially in chromosome 4. Salivary gland chromosomes of F₁ larvae reared either from wild-caught females or wild-caught males mated to standard laboratory stocks were examined for gene arrangements. *Drosophila bostrycha* and *D. affinidisjuncta* share a polymorphism for inversion 4v, which is much more frequent in the latter than in the former. In *D. disjuncta* 4v has been found only joined in a haplotype with three other inversions ($g^2 h^2 i^2$) at a low frequency at Kipahulu Valley. *Drosophila disjuncta* is unique in having another fourth chromosome inversion, 4k, which is highest in frequency at Waikamoi. A new inversion, 2s, was discovered at Uluini Stream. Interspecific hybridizations were carried out in small mass matings. Backcrosses and dissections demonstrated that all F₁ females were fertile. All F₁ males were sterile in either of two categories with reciprocal hybrids uniformly manifesting one or the other type. Attempts to model the sterility mechanism suggest that more than chromosomal sterility is involved. Our results are discussed in relation to other closely related clusters of species having heterochromatin-based karyotype variations.

Key words: heterochromatin, hybrid sterility, inversion, polymorphism, species divergence.

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Trois espèces allopatriques des Îles Hawaï, *Drosophila bostrycha* (Molokai), *D. affinidisjuncta* (Maui, ouest) et *D. disjuncta* (Maui, est) sont très semblables dans leur morphologie, mais elles diffèrent au niveau des chromosomes en métaphase par la quantité et la distribution de l'hétérochromatine. Leurs chromosomes polytènes sont virtuellement homoséquentiels, n'offrant que d'infimes différences à l'extrémité des microchromosomes. Chacun d'eux est polymorphe en fonction d'une ou de plusieurs inversions, en particulier le chromosome 4. Les chromosomes de la glande salivaire des larves de la F₁, dont l'élevage provient soit de mâles ou de femelles sauvages capturés et accouplés avec les stocks standard de laboratoire, ont été examinés pour en étudier la disposition des gènes. *Drosophila bostrycha* et *D. affinidisjuncta* ont en commun un polymorphisme pour l'inversion 4v, laquelle est plus fréquente cependant chez la seconde espèce. Chez *D. disjuncta*, la 4v n'a été trouvée que chez un haplotype possédant trois autres inversions ($g^2 h^2 i^2$) de faibles fréquences, provenant de la vallée de Kipahulu. *Drosophila disjuncta* est unique, en ce qu'elle possède une autre inversion du quatrième chromosome, la 4k, laquelle présente la plus haute fréquence à Waikamoi. Une nouvelle inversion, la 2s, a été découverte à Uluini Stream. Des hybridations interspécifiques ont été obtenues suite à des accouplements par petits groupes. Des rétrocroisements et des dissections ont démontré que toutes les femelles F₁ étaient fertiles. Tous les mâles F₁ étaient stériles chez l'une ou l'autre des catégories dont les hybrides réciproques manifestaient uniformément l'un ou l'autre type. Des tentatives de modélisation du mécanisme de stérilité suggèrent que la stérilité chromosomique n'est pas seule à être impliquée. Nos résultats sont discutés en fonction d'autres groupes étroitement reliés qui présentent des variations de caryotypes basées sur l'hétérochromatine.

Mots clés: hétérochromatine, stérilité des hybrides, inversion, polymorphisme, divergence des espèces.

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Introduction

Evolutionary studies of the species belonging to the family Drosophilidae endemic to the Hawaiian Archipelago have contributed greatly toward the understanding of speciation. Because the Hawaiian Islands are of recent geologic formation, the events of species formation on these islands have been likewise recent. Concentrating on the members of the picture-winged group of Hawaiian *Drosophila*, Carson and co-workers (reviewed in Carson and Yoon 1982) have documented, from morphological, cytological, and geographical distribution studies, the existence of some 103 species showing various degrees of divergence.

Distinct phylogenetic relationships have been postulated among the picture-winged *Drosophila* based on the analysis of

polytene chromosomes (Carson and Kaneshiro 1976; Carson and Yoon 1982). Within the *D. grimshawi* subgroup is an especially interesting cluster of three closely related species, *D. bostrycha*, *D. disjuncta*, and *D. affinidisjuncta*, which are virtually homosequential (Carson and Stalker 1968; Clayton *et al.* 1972); that is, they share a standard sequence of polytene chromosome bands (Carson and Kaneshiro 1976). These species share a polymorphism for a fourth chromosome inversion (4v) (Carson and Sato 1969). Morphologically, the three are very similar (Hardy 1978). Each is endemic to one of three separate volcanic domes, two on the island of Maui and one on the island of Molokai. Further study of this species cluster may provide additional information on species differentiation and suggest modes of speciation for insular organisms. Presented here are the results of chromosome analyses and hybridization experiments of this species cluster.

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TABLE 1. Locations and dates of *Drosophila* from Molokai and Maui

Species	Location	Collection dates	Collection code
<i>D. bostrycha</i>	South Hanalilolilo, Central Molokai, 920 m	July 9–11, 1973	S-16
		January 9–11, 1975	T-38*
		April 8–9, 1975	T-46
		July 15–19, 1975	T-60
<i>D. affinisdisjuncta</i>	Apee, East Molokai, 520 m	September 24–26, 1973	S-30
		July 5–7, 1973	S-15*
	Kaulalewelewe, West Maui, 920 m	September 26–28, 1973	S-36
		April 10–12, 1975	T-47
<i>D. disjuncta</i>	Hanaula, West Maui, 1220 m		
	Waikamoi, East Maui, 1220 m	April 9–10, 1974	S-69
	Waikamoi, East Maui, 1070 m	April 9–10, 1974	S-72
	Waikamoi, East Maui, 1070 m	October 24–26, 1974	T-27*
	Waikamoi, East Maui, 1170 m	April 9–10, 1974	S-70
	Uluini Stream, East Maui, 490 m	November 23–24, 1973	S-48
	Kipahulu Valley, East Maui, 670 m	January 14–15, 1974	S-56

*used only in hybridization crosses.

Materials and methods

Samples of the three species were collected from various populations within the known geographic distributions (Table 1). *Drosophila bostrycha* is endemic to central and eastern Molokai (Fig. 1). *Drosophila disjuncta* inhabits the rainforests on the slopes of Haleakala Crater, East Maui, ranging from Waikamoi in the north to Kipahulu Valley in the southeast (Carson and Sato 1969). *Drosophila affinisdisjuncta* is known only from West Maui (Hardy 1978; Baimai and Ahearn 1978). These three species oviposit and feed as larvae upon two endemic host plants, *Freyinetia* and *Clermontia*, which are quite common on Molokai and Maui (Montgomery 1975).

Males and females from the field were isolated and the F_1 subjected to cytological study in the laboratory. F_1 larvae were reared from each female (isofemale line). Wild-caught males were crossed individually to virgin females from a laboratory stock with known band sequences. Acetoorcein squash preparations of salivary gland chromosomes were made, polytene band sequences were observed under the light microscope, and frequencies of arrangements calculated according to the method of Carson and Sato (1969). Methods for examination of metaphase chromosomes have been described in Baimai (1977). Chromosomes in mitotic anaphase were studied to determine the position of the centromeres. Anaphases were obtained from third instar larval ganglia dissected in 0.7% saline, swelled in 1% sodium citrate, fixed in Carnoy's 3:1, stained in 2% acetoorcein, and squashed in 45% acetic acid.

Hybridizations were made in nearly all possible combinations between the seven populations of the three species (Fig. 2). Laboratory stocks of *D. bostrycha*, *D. affinisdisjuncta*, and *D. disjuncta* were derived from individual wild-caught females. These isofemale lines were cultured at 20° C by standard methods developed for Hawaiian *Drosophila* (Wheeler and Clayton 1965). Adult flies were separated by sex after eclosion and aged to sexual maturity (3 to 5 weeks). Hybridizations were performed in small mass matings of three to five pairs per vial. Parents were brooded weekly. Wheeler–Clayton medium in the mating vials was supplemented with *Clermontia* juice (water extract of *Clermontia* leaves) and small pieces of rotted *Freyinetia* bark to make conditions favorable for oviposition.

F_1 hybrid adults were collected after eclosion, aged, and subsequently tested for fertility. F_1 males (aged at least 4 weeks) were dissected and the testes examined microscopically for the status of spermatogenesis. F_1 males consistently fell into either of two classes of sterility. These classes were distinguished by the stage of spermatogenesis at which sperm development was arrested as well as general morphology of the testes (adopted from Craddock 1975). Sterility class I was characterized by presence of cells and cell debris within testes of reduced size and with reduced number of coils. Sterility class II was

typified by presence of all stages of spermatogenesis within a testis of normal morphology but absence of any mature, motile sperm within the vasa deferentia.

F_1 hybrid females were backcrossed to males of the parental populations. Flies were transferred to fresh food vials weekly and old vials were examined for the presence of larvae. Crosses were allowed to proceed for a maximum of 8 weeks; then the females were dissected and the ovaries and sperm storage organs examined microscopically for presence of mature eggs and sperm, respectively.

Results

Polytene chromosome analysis

Detailed analysis of polytene chromosome band sequences revealed population as well as species differences. Rearrangements of the standard band sequences (i.e., the polytene band sequences of *D. grimshawi*) were observed primarily in chromosome 4 (Table 2). Each population of the three species sampled carried the standard arrangement at frequencies ranging from 100% in *D. bostrycha* at Apee to 15% in *D. affinisdisjuncta* from Hanaula.

The inversion of band sequence on the fourth chromosome called 4v (described by Carson and Stalker 1968) is seen in all three species. This inversion is most common in *D. affinisdisjuncta*, being present in 78.4% of fourth chromosomes at Kaulalewelewe and in 85% of fourth chromosomes at Hanaula. *Drosophila bostrycha* from South Hanalilolilo had 4v at a frequency of 51.2%. However, *D. bostrycha* collected from Apee did not carry the 4v inversion. In *D. disjuncta*, 4v appeared only in combination with three additional and overlapping inversions, g^2 , h^2 , and i^2 (breakpoints described in Carson 1969). This complex set of inversions occurs only in the Kipahulu Valley population as an unrecombined haplotype having a frequency of 1.9%. However, at 915 m, a higher elevation than our sampling location, this complex was present at an average frequency of 15.8% (Carson and Sato 1969).

All populations of *D. disjuncta* sampled exhibited inversion 4k (described in Carson 1969) which is absent from *D. bostrycha* and *D. affinisdisjuncta*. The frequency of inversion 4k varies from 0.5% in the Kipahulu Valley population to 80.4% in the higher altitude Waikamoi population. A new rearrangement of chromosome 2, denoted as 2s (Fig. 3), was found in one of the 36 chromosomes examined from the Uluini Stream population. The breakpoints of this inversion are mapped in

FIG. 1. Geographic distributions, metaphase karyotypes, and sali vary gland chromosome gene arrangements of *D. bostrycha* (Molokai), *D. affinisdisjuncta* (West Maui), and *D. disjuncta* (East Maui).

D. bostrycha				D. affinisdisjuncta		D. disjuncta		
		Molokai		West Maui		East Maui				
		South Hanalei	Apee	Kaulalewelewe	Hanalei	Waikamoi	Uluini Stream	Kipahulu Valley
D. bostrycha								
		Molokai								
		South Hanalei		3	3					
		Apee	3		4	6	14	5	2	
		Kaulalewelewe	4	4		10	8	3	1	
		Hanalei		6	6		9	7	12	
		Waikamoi		1	2	9		7	9	
		Uluini Stream		5	2	7	6		9	
		Kipahulu Valley		7	13	8	8	9		

FIG. 2. Matrix of intra- and inter-specific hybridizations. Within each square is the number of small mass matings set up for the cross. Empty squares are crosses which were not made.

Carson 1983. The second chromosome of all other species' populations was monomorphic.

The polytene salivary gland chromosomes of F_1 interspecific hybrid larvae have also been examined. All major chromosome arms are completely synapsed. The exception is chromosome 6, the microchromosome or dot in mitotic metaphases. *Drosophila*

disjuncta has a few additional small bands at the distal tip of chromosome 6, which are absent from *D. bostrycha* and *D. affinisdisjuncta*. In F_1 hybrids of the latter two species, the band sequence of chromosome 6 is identical and the homologues synapse (Fig. 4a). In contrast, chromosome 6 in F_1 hybrids of *D. disjuncta* and *D. bostrycha* or *D. affinisdisjuncta* shows some asynapsis (Figs. 4b and 4c). The additional bands of the *D. disjuncta* homologue are very evident in these hybrids.

The chromosomes of the three species as they appear at mitotic anaphase are presented in Fig. 5. These are all female complements so no conclusion can be made about the Y chromosome. However, it is clear that all the major autosomes and the X chromosome are acrocentric.

Intraspecific hybridizations

Crosses between populations within each species were easily obtained. In every case F_1 females were fertile as assessed by backcrosses to parental population males and dissections of reproductive systems (Table 3). F_1 adult sex ratios were measured at eclosion (Table 4). Males and females appeared in roughly equal numbers in *D. bostrycha*. The sex ratio in interpopulation F_1 hybrids of *D. affinisdisjuncta* was in favor of females. Among the four *D. disjuncta* interpopulation crosses examined there was significant variation in sex ratio as indicated by the heterogeneity χ^2 test ($\chi^2 = 20.471$, $p = 0.01$). However, if the most aberrant cross (Waikamoi $\times$ Uluini Stream) is removed from the data, the heterogeneity becomes nonsignificant ($\chi^2 = 2.502$, $p = 0.2-0.3$) and the χ^2 of the pooled data borders on significance ($\chi^2 = 3.608$, $p = 0.05-0.1$). Thus, the interpopulation F_1 hybrids tend to have a deviant sex ratio in favor of females in *D. disjuncta*.

Reproductive systems of mature (approximately 4 week old) F_1 males were examined for each intraspecific cross (Fig. 6). All crosses yielded fertile males. However, a small but significant number of *D. affinisdisjuncta* males from the Hanalei $\times$ Kaulalewelewe cross were sterile, lacking any mature, motile sperm. Within the *D. disjuncta* interpopulation crosses, two

TABLE 2. Frequencies (%) of fourth chromosome gene sequences in populations of *D. bostrycha*, *D. affinisdisjuncta*, and *D. disjuncta*

Species and locality	No. of individuals tested		No. of chromosomes examined	Arrangement			
	♀ ♀	♂ ♂		4+	4k	4v	4vg ² h ² i ²
<i>D. bostrycha</i>							
South Hanalilolilo (S16, T46, T60)	14	12	80	48.8	—	51.2	—
Apee (S30)	13	—	52	100.0	—	—	—
<i>D. affinisdisjuncta</i>							
Kaulalewelewe (S36)	29	9	134	21.6	—	78.4	—
Hanaula (T47)	3	4	20	15.0	—	85.0	—
<i>D. disjuncta</i>							
Waikamoi (S69, S70)	12	2	51	19.6	80.4	—	—
Waikamoi (S72)	7	6	37	27.0	73.0	—	—
Uluini Stream (S48)	6	6	36	97.2	2.8	—	—
Kipahulu Valley (S56)	38	30	212	97.6	0.5	—	1.9

FIG. 3. Heterozygous inversion 2s/+ from the Uluini Stream population of *D. disjuncta*.

resulted in a percentage of males that were class II sterile. In both cases the cross involved males of the Uluini Stream population and females of another population. When the stock used for these crosses was tested against another isofemale line from Uluini Stream, all F_1 males were fertile ($n = 26$).

Interspecific hybridizations

Hybridizations of the three species were obtained with varying degrees of difficulty. F_1 hybrid females, however, were fertile (Table 5). In backcrosses nearly every mating yielded BC_1 larvae. Dissections showed that F_1 females had completely normal reproductive systems and in most cases had been inseminated.

Male and female F_1 hybrids of *D. bostrycha* and *D. affinisdisjuncta* appear in approximately equal numbers (Table 6). Likewise the cross *D. bostrycha* $\times$ *D. disjuncta* and its reciprocal yield progenies with a normal sex ratio. However,

when *D. affinisdisjuncta* females are crossed with male *D. disjuncta* there is a significant deviation in the F_1 sex ratio in favor of females. Among progenies of the cross female *D. disjuncta* $\times$ male *D. affinisdisjuncta* is a significant amount of heterogeneity in the sex ratio. If the cross Kipahulu Valley $\times$ Hanaula is removed from the data set, the heterogeneity becomes nonsignificant ($\chi^2 = 2.212$, $p = 0.7$) and the sex ratio for the remaining progenies is small ($\chi^2 = 0.112$, $p = 0.5-0.7$).

Dissections of F_1 interspecific hybrid males revealed what is the most striking result of these hybridizations (Fig. 6). All male progeny of interspecific crosses were sterile. All F_1 males from a particular cross were uniform in the sterility they displayed; they were either class I or class II sterile. If the progenies are considered in pairs with reciprocal hybridizations comprising each pair, then F_1 males of one hybridization are SI and F_1 males of the reciprocal hybridization are SII. For example, F_1 males from the cross female *D. affinisdisjuncta* (Kaulalewelewe) $\times$

FIG. 4. Salivary gland polytene chromosome 6 of F_1 interspecific hybrid larvae. (a) *Drosophila affinidisjuncta* ♀ × *D. bostrycha*, ♂. Homologues are well synapsed and cannot be distinguished. (b) *Drosophila disjuncta* ♀ × *D. bostrycha* ♂. (c) *Drosophila affinidisjuncta* ♀ × *D. disjuncta* ♂. Chromosome 6 of *D. disjuncta* with characteristic extra band at the distal tip (arrow) is shown on the left in each figure.

TABLE 3. Intraspecific F_1 hybrid female fertility

Population A ♀♀ × population B ♂♂	Backcrosses					
	F_1 ♀♀ × A ♂♂			F_1 ♀♀ × B ♂♂		
	Success ratio*	Dissections		Success ratio	Dissections	
		Total	No. inseminated		Total	No. inseminated
<i>D. bostrycha</i>						
Apee × South Hanalilolilo	F		F			
South Hanalilolilo × Apee	F			F		
<i>D. affinidisjuncta</i>						
Kaulalewelewe × Hanaula	7/7	21	21	7/7	20	20
Hanaula × Kaulalewelewe	7/7	20	20	7/7	21	18
<i>D. disjuncta</i>						
Waikamoi × Uluini Stream	7/8	25	17	7/7	21	21
Uluini Stream × Waikamoi	1/3	6	4	0/3	4	3
<i>D. disjuncta</i>						
Uluini Stream × Kipahulu Valley	6/7	17	11	7/7	18	15
Kipahulu Valley × Uluini Stream	7/7	18	16	7/7	14	10
Kipahulu Valley × Waikamoi	7/7	20	14	7/7	20	15
Waikamoi × Kipahulu Valley	5/7	21	11	5/7	16	12

*Success ratio is the number of small mass matings yielding larvae/total number of matings. F, F_1 ♀♀ fertile based on presence of larvae in mating vials (detailed records not kept).

male *D. disjuncta* (Kipahulu Valley) are class I sterile while F_1 males from the reciprocal cross female *D. disjuncta* (Kipahulu Valley) × male *D. affinidisjuncta* (Kaulalewelewe) are class II sterile. Likewise, the cross female *D. disjuncta* (Kipahulu Valley) × male *D. bostrycha* (Apee) yields F_1 males with class I sterility while the reciprocal hybridization generates F_1 males of class II sterility.

An even more general pattern emerges when the entire matrix of hybridizations is examined. All interspecific crosses using *D. affinidisjuncta* as the female parent produce F_1 males having class I sterility and all interspecific crosses using *D. bostrycha* as the male parent produce F_1 males having class I sterility.

Discussion

The present investigation has led to more questions than answers. D. E. Hardy, world authority on Hawaiian *Drosophila* systematics, recognized that specimens collected from Molokai and Maui were closely related to *D. grimshawi*. He described two species, one from Molokai and one from Maui, in 1965. He differentiated *D. bostrycha* from *D. grimshawi* by the yellow color of the femur of the male forelegs. *Drosophila disjuncta* differed from *D. bostrycha*, as Hardy saw it, only in that *D. bostrycha* males have luxurious ciliation of the tibia on the forelegs, which *D. disjuncta* males lack (Hardy 1965).

Some years later, Baimai and Ahearn (1978) discovered that

FIG. 5. Chromosomes at mitotic anaphase. (a) *Drosophila disjuncta* ♀, (b) *D. bostrycha* ♀, and (c) *D. affinisdisjuncta* ♀. The dot chromosomes are not always visible. Original magnification 1200 ×.

the West Maui populations that had previously been lumped under the name *D. disjuncta*, had substantial genetic differences from the populations of East Maui. All five major metaphase chromosomes show heterochromatic blocks, near the centromeres, that are very much longer than those of either *D.*

		♀ ♂	<i>D. bostrycha</i>		<i>D. disjuncta</i>			<i>D. affinisdisjuncta</i>	
			Molokai		East Maui			West Maui	
			South Hanalei	Apee	Waikamoi	Ukuni Stream	Kipahulu Valley	Kaulaewelewe	Hanalei
<i>D. bostrycha</i>	South Hanalei			F n=20	—	—	—	SI n=19	—
	Apee		F n=15		SI n=30	SI n=20	SI n=26	SI n=18	SI n=26
<i>D. disjuncta</i>	Waikamoi		—	SII n=26		F n=26	F n=27	SI n=27	SI n=25
	Ukuni Stream		—	SII n=21	F n=69 (17.4% SI)		F n=40 (12.5% SI)	SI n=8	SI n=26
	Kipahulu Valley		—	SII n=35	F n=26	F n=26		SI n=33	SI n=26
<i>D. affinisdisjuncta</i>	Kaulaewelewe		SII n=16	SII n=26	SII n=31	SII n=12	SII n=24		F n=138 (5.8% SI)
	Hanalei		—	SII n=20	SII n=27	SII n=27	SII n=52	F n=34	

FIG. 6. Summary of F_1 male fertility in intra- and inter-specific hybridizations. F, fertile; SI, class I sterility (block during spermatogenesis); SII, class II sterility (block occurs in spermiogenesis); n, number of males dissected.

bostrycha or the East Maui populations of *D. disjuncta* (see Fig. 1 in Baimai and Ahearn 1978; Fig. 1 in the present paper gives a diagrammatic representation of these karyotypes). Except for the curious fourth chromosome ($4vg^2h^2i^2$) of Kipahulu valley (Baimai 1975a), these karyotypes are invariable within each species; any single metaphase from any one of the three species is identifiable as belonging to that species without exception. Furthermore, F_1 males that are hybrids between species were invariably sterile. In view of the preliminary data presenting these facts, Hardy (1978) described the West Maui populations as a third species, *affinisdisjuncta*, stating that it was morphologically indistinguishable from *disjuncta*. This systematic judgment is amply born out by the new data presented in the present paper.

The divergence of these three species must have occurred comparatively recently. The Maui Island complex, to which they are endemic, has had an unique geological history. East Molokai, in relative terms the oldest at 1.3 to 1.5 million years, is separated from the younger West Maui, aged 1.15 to 1.3 million years, by a shallow channel (244 m) only 14 km in width. Haleakala Crater, the volcanic dome of East Maui, is the youngest at 0.4 to 0.8 million years. It is separated from West Maui by a saddle only 38 m above sea level and 30 km in width. The entire group of islands (Maui, Molokai, Lanai, and Kahoolawe) has undergone at least two cycles of separation and rejoining because of changes in sea level during the Pleistocene period. Final geographic isolation of Molokai and East and West Maui occurred in the recent geologic past (Macdonald and Abbott 1970).

The standard polytene chromosome band sequence, that of *D. grimshawi*, is found in all three species. However, the

TABLE 4. Sex ratios in F₁ progenies of intraspecific crosses

Population A ♀♀ × population B ♂♂	No. of F ₁ adults		df	χ ²	p
	♀♀	♂♂			
<i>D. bostrycha</i>					
Apee × South Hanalilolilo	20	24			
South Hanalilolilo × Apee	19	22			
χ ² analysis					
Total			2	0.584	
Heterogeneity			1	0.008	>0.9
Pooled	39	46	1	0.576	0.3–0.5
<i>D. affinidisjuncta</i>					
Kaulalewelewe × Hanaula	91	78			
Hanaula × Kaulalewelewe	220	179			
χ ² analysis					
Total			2	5.213	
Heterogeneity			1	0.075	0.7–0.8
Pooled	311	257	1	5.138	<0.05
<i>D. disjuncta</i>					
Waikamoi × Uluini Stream	304	172			
Uluini stream × Waikamoi	227	196			
Uluini Stream × Kipahulu Valley	247	249			
Kipahulu Valley × Uluini Stream	240	199			
χ ² analysis					
Total			4	42.72	
Heterogeneity			3	20.471	<0.01
Pooled	1018	816	1	22.249	<0.01

frequency of the standard varies considerably between species and even between populations within a species. Curiously, the standard fourth chromosome of *D. affinidisjuncta* is differentiated from the standard in the other species by the presence of two permanent constrictions (4B and 4C) at precise points (Carson 1983, Table 1). It is not known whether these constrictions are associated with nucleolar organizers. This is further evidence of the uniqueness of *affinidisjuncta*. In *D. bostrycha* at Apee the standard sequence is nearly fixed. The standard arrangement is also nearly fixed in *D. disjuncta* at Uluini Stream and Kipahulu Valley (Table 2). However, in *D. affinidisjuncta* only 15 (Hanaula) to 21.6% (Kaulalewelewe) of the haploid genomes are standard. Perusal of Table 2 reveals that *disjuncta* is the most polymorphic of the species with regard to inversions; indeed each of the three populations studied are different from one another, an observation previously suggested by the data of Carson and Sato (1969). Although Carson (1983) states that chromosome 6, the dot, is invariant for polytene band sequence in the Hawaiian picture wings, F₁ hybrids involving *D. disjuncta* show that this species has additional terminal bands (Fig. 4).

An early survey of electrophoretic variation in natural populations of the Hawaiian picture wings by Rockwood *et al.* (1971) included the three species studied here. However, at that time *D. affinidisjuncta* was not recognized as such but was called the West Maui population of *D. disjuncta*. The average heterozygosity, based on only six loci, was 0.067 for *D. disjuncta* (the Kipahulu Valley population) and 0.150 and 0.166 for the Kaulalewelewe and Hanaula populations of *D. affinidisjuncta*, respectively. Their results indicated that phenotypic frequencies for the esterase-B locus were significantly different between *D. disjuncta* and *D. affinidisjuncta*. We briefly surveyed electrophoretic variants of some enzymes and found

species differences for malate dehydrogenase (MDH), which is a dimeric protein. *Drosophila bostrycha* is fixed for a "slow-migrating" electromorph, which it has in common with *D. grimshawi*. *Drosophila disjuncta* is fixed for a "fast-migrating" electromorph.

Drosophila bostrycha and *D. disjuncta* were included in Ahearn and Kuhn's (1981) histochemical study of aldehyde oxidase distribution patterns in larval tissues and mature imaginal discs. Significant differences in overall activity were seen in the larval hypoderm, salivary glands, and the eye-antennal imaginal disc. With the antennal and leg discs, correlate areas of AO activity were seen in every species examined. Both *bostrycha* and *disjuncta* had activity in the presumptive first antennal segment and the presumptive coxa and, in addition, *disjuncta* also had activity in the presumptive third antennal segment and the presumptive proximal tibia-femur.

When we discovered the karyotype differences between *bostrycha*, *disjuncta*, and *affinidisjuncta*, we were unable, for lack of technique, to determine whether the heterochromatic additions to each chromosome have been accompanied by a change in the position of the centromere (Baimai and Ahearn 1978). Thus the karyotypes for *bostrycha* and *affinidisjuncta* were described in terms of J's and V's and this was reiterated in Table 2 of Carson's 1981 review of homosequential species. However, from our anaphase spreads of each species (Fig. 5), it is clear that the X and major autosomes are acrocentric.

There are many karyotype alterations owing to heterochromatic changes reported in the *Drosophila* literature. At least five examples of karyotype differentiation are known between populations within species and these are due to additions of heterochromatin to the dot, the Y, and sometimes the X. Baimai *et al.* (1983) described six karyotypes for *D. serido* and five

TABLE 5. Interspecific F₁ hybrid female fertility

Species A ♀♀ × species B ♂♂	Backcrosses					
	F ₁ ♀♀ × A ♂♂			F ₁ ♀♀ × B ♂♂		
	Success ratio*	Dissections		Success ratio	Dissections	
		Total	No. inseminated		Total	No. inseminated
<i>D. bostrycha</i> × <i>D. affinisdisjuncta</i>						
South Hanalilolilo × Kaulalewelewe	F			F		
Apee × Kaulalewelewe	F			F		
Apee × Hanaula	7/7	20	19	7/7	20	20
<i>D. affinisdisjuncta</i> × <i>D. bostrycha</i>						
Kaulalewelewe × South Hanalilolilo	F			F		
Kaulalewelewe × Apee	F			F		
Hanaula × Apee	7/7	20	19	7/7	20	20
<i>D. affinisdisjuncta</i> × <i>D. disjuncta</i>						
Kaulalewelewe × Waikamoi	6/7	18	11	5/7	18	14
Kaulalewelewe × Uluini Stream	F			F		
Kaulalewelewe × Kipahulu Valley	6/7	15	12	6/7	21	17
Hanaula × Waikamoi	7/7	21	17	7/7	19	19
Hanaula × Uluini Stream	7/7	21	21	7/7	21	20
Hanaula × Kipahulu Valley	7/7	17	17	7/7	19	18
<i>D. disjuncta</i> × <i>D. affinisdisjuncta</i>						
Waikamoi × Kaulalewelewe	6/6	25	23	6/6	27	27
Uluini Stream × Kaulalewelewe	F			F		
Kipahulu Valley × Kaulalewelewe	3/4	6	3	5/5	12	9
Waikamoi × Hanaula	7/7	20	18	7/7	22	22
Uluini Stream × Hanaula	5/5	15	15	7/7	20	20
Kipahulu Valley × Hanaula	7/7	20	20	7/7	20	20
<i>D. bostrycha</i> × <i>D. disjuncta</i>						
Apee × Waikamoi	5/7	17	14	5/7	13	10
Apee × Uluini Stream	F			F		
Apee × Kipahulu Valley	6/7	20	15	7/7	17	17
<i>D. disjuncta</i> × <i>D. bostrycha</i>						
Waikamoi × Apee	5/5	9	8	5/5	14	13
Uluini Stream × Apee	F			F		
Kipahulu Valley × Apee	6/6	15	6	7/7	21	21

* Success ratio is the number of small mass matings yielding larvae/total number of matings. F, F₁ ♀♀ fertile based on presence of larvae in mating vials (detailed information not available).

karyotypes for *D. meridionalis* both of the *repleta* group in Brazil. No hybridizations between karyotypic strains were attempted. *Drosophila paranaensis*, also of the *repleta* group and endemic to Central and South America, was found to have three karyotypic strains which differ by additions of heterochromatin to the dot (Wasserman and Wilson 1957; reviewed by Wasserman 1982). Thus the dot of strain "a" becomes a small V in strain "b" and a large V in strain "c." Hybridizations within strains yielded fertile F₁ progeny but between strains "a" and "c" yielded sterile F₁ males.

In the *melanogaster* group, *D. birchii* is endemic to Northern Australia and Papua New Guinea. Baimai (1969) described seven karyotypic strains which differ by additions of heterochromatin to the dot, the X, and the Y from the basic karyotype of two V's, one J, and one dot. His hybridization studies (Baimai 1970) included only four of the seven strains but revealed that only one of these, collected both in Cairns, Australia, and in Rabaul, Island of New Britain, New Guinea, gave sterile F₁ males when hybridized with the other three strains. The Rabaul strain also appeared to be differentiated from the others by courtship behaviour. A subcosmopolitan species ranging from the Orient and Australia across the Pacific

to South America, *D. kikkawai* in the *melanogaster* group has seven karyotypic strains (Baimai and Chumchong 1980). Siblings of *D. kikkawai*, *D. leontia*, and *D. bocki*, endemic to Thailand and Taiwan, share a karyotype of two V's, one R and one dot. In *D. kikkawai* this karyotype is expanded by heterochromatin additions to the dot and the Y; in fact, the dot is never seen. Cursory hybrid studies showed that the strains from Singapore and Brazil yield fertile F₁ hybrids but that a strain from Sabah, North Borneo, is sexually isolated from the Singapore strain.

None of the previous findings have led to new species designations as our findings did. However, there are two examples of close karyotype relationships being found for species already described on morphological and biogeographical bases. In the *immigrans* group, *D. nasuta* of the Seychelles has a karyotype of two R's, one V, and one dot. The karyotype of very close relatives, *D. kepulauan* of Borneo and the Philippines and *D. albomicans* of Thailand and Taiwan, differ by heterochromatin additions to the dot and the Y. In addition, the X and Y have fused with one of the rod-shaped autosomes in *D. albomicans*. Only F₂ interspecific hybrids involving *D. albomicans* show partial sterility because of

TABLE 6. Sex ratios in F₁ progenies of intraspecific hybridizations

Species A ♀♀ × species B ♂♂	No. of F ₁ adults		df	χ ²	p
	♀♀	♂♂			
<i>D. bostrycha</i> × <i>D. affinidisjuncta</i>					
South Hanalilolilo × Kaulalewelewe	14	16			
Apee × Kaulalewelewe	54	66			
χ ² analysis					
Total			2	1.333	
Heterogeneity			1	0.026	0.8–0.9
Pooled	68	82	1	1.307	0.2–0.3
<i>D. bostrycha</i> × <i>D. disjuncta</i>					
Apee × Waikamoi	69	72			
Apee × Uluini Stream	52	46			
χ ² analysis					
Total			2	0.431	
Heterogeneity			1	0.398	0.5–0.7
Pooled	121	118	1	0.038	0.8–0.9
<i>D. disjuncta</i> × <i>D. bostrycha</i>					
Waikamoi × Apee	48	41			
Uluini Stream × Apee	101	90			
Kipahulu Valley × Apee	50	43			
χ ² analysis					
Total			3	1.712	
Heterogeneity			2	0.036	> 0.9
Pooled	199	174	1	1.676	0.1–0.2
<i>D. affinidisjuncta</i> × <i>D. bostrycha</i>					
Kaulalewelewe × South Hanalilolilo	26	23			
Kaulalewelewe × Apee	36	32			
χ ² analysis					
Total			2	0.419	
Heterogeneity			1	0.00	> 0.9
Pooled	62	55	1	0.419	0.5–0.7
<i>D. affinidisjuncta</i> × <i>D. disjuncta</i>					
Kaulalewelewe × Waikamoi	68	56			
Kaulalewelewe × Uluini Stream	7	9			
Hanaula × Waikamoi	146	139			
Hanaula × Uluini Stream	179	140			
Hanaula × Kipahulu Valley	202	137			
χ ² analysis					
Total			5	18.814	
Heterogeneity			4	5.295	0.2–0.3
Pooled	602	481	1	13.519	<0.01
<i>D. disjuncta</i> × <i>D. affinidisjuncta</i>					
Waikamoi × Kaulalewelewe	151	134			
Uluini Stream × Kaulalewelewe	16	12			
Kipahulu Valley × Kaulalewelewe	28	30			
Waikamoi × Hanaula	59	53			
Uluini Stream × Hanaula	271	285			
Kipahulu Valley × Hanaula	269	152			
χ ² analysis					
Total			6	34.843	
Heterogeneity			5	23.121	<0.01
Pooled	794	666	1	11.222	<0.01

chromosome imbalance. There was no measureable mating behavior differentiation (Wilson *et al.* 1969; Kitagawa 1978). Four cactus-breeding species endemic to the Mexican Sonoran desert were initially placed into different subgenera based solely on morphology. However, Ward and Heed (1970) discovered that *D. pachea*, *D. acanthoptera*, *D. nannoptera*, and a species

yet to be named shared most of their polytene band sequences and differed karyotypically by additions of heterochromatin to the dot, the Y, and in *nannoptera* to the rod-shaped autosome creating a V. The *nannoptera* group was erected to accommodate these four. No hybrids were obtained between any of the species since no matings occurred.

The basic karyotype for Hawaiian picture-winged *Drosophila* is five rods and a dot (5R1D). Yoon and Richardson (1978) review six departures from this state owing to additions of heterochromatin to the dot. These are *D. heedi*, *D. lasiopoda*, *D. limitata*, *D. uniseriata*, *D. psilophallus*, and *D. montgomeryi*. Of these only one, *D. uniseriata* of Oahu, has been hybridized to close relatives. Yang and Wheeler (1969) obtained fertile F₁ females but sterile F₁ males from both reciprocal crosses to *D. paucipuncta* of Hawaii. They were unable to get matings between *uniseriata* and *D. punalua* of Oahu. Three additional species show karyotype departures from 5R1D. They are *D. prostopalpis* with 4R1V1D (Clayton 1971), *D. melanocephala* with 5R1V (Clayton 1969; Clayton *et al.* 1972), and *D. cyrtoloma* with 5V1I (Clayton 1969). The last case is a striking example in which all major elements are enlarged with heterochromatin but, unlike the subjects of this paper, the heterochromatin creates an extraordinary set of additional chromosomal arms (Clayton 1985). Among these, attempts were made to hybridize *melanocephala* to a close relative, *D. hemipeza*, but no matings could be obtained (Yang and Wheeler 1969).

Besides *bostrycha*, *disjuncta*, and *affinidisjuncta*, there are seven additional picture wings with heterochromatic variations that do not lead to a departure from the 5R1D karyotype. *Drosophila digressa* was described by Clayton (1976) as having larger rods and dot than close relatives. Doubling in size of one of the rods occurs in *D. orphnopeza* and *D. gymnobasis* (Clayton 1969, 1971). This is also true for the Laupahoehoe population of *D. setosimentum* but this condition is a polymorphism within this species (Clayton *et al.* 1972). A similar heterochromatin polymorphism has been studied in *D. silvarientis*, which also has enlarged dots (Clayton 1969).

Baimai (1975a, 1975b, 1977) carefully investigated similar heterochromatin polymorphisms in *D. disjuncta*, *D. formella*, and *D. recticilia*. He found that in each case the additional heterochromatin lengthening of the rods occurred on a chromosome in which inversions had a breakpoint in the centric heterochromatin. For example, in *D. disjuncta* the $4vg^2h^2i^2$ inversions, which we have discussed earlier, are correlated with increased chromosome length. The h^2 and i^2 right breaks are both in the centric heterochromatin. Baimai hypothesized that centric inversion breakpoints may provide a mechanism for excessive replication of heterochromatin and thereby heterochromatin addition. This, indeed, may be true for some situations. However, such a mechanism cannot explain the origin of the heterochromatin blocks in *bostrycha* and *affinidisjuncta* since they are homosequential.

We have made the most exhaustive hybridization studies undertaken for Hawaiian picture wings with the exception of Ohta's (1980) work on strains of *D. grimshawi*. Yang and Wheeler (1969) had included *bostrycha* and *disjuncta* in their experiments. They reported obtaining fertile F₁ females and sterile F₁ males but, although the hybrids were dissected, no description of the male sterility was offered.

Results of our intraspecific crosses indicate some genetic differentiation between the populations of *affinidisjuncta* since the sex ratio is skewed in favor of females (Table 4) and some F₁ male sterility is seen (Fig. 6). For similar reasons we conclude that the Uluni Stream population of *disjuncta* is diverging from the others.

The only interspecific hybridization that resulted in an aberrant sex ratio was between *affinidisjuncta* females and *disjuncta* males where F₁ females were in excess (Table 6). All F₁ hybrid females were fertile (Table 5). All F₁ hybrid males

were sterile (Fig. 6) and, as has been reported in the Results, the block in sperm development occurs at one of two stages depending upon the female and male parents. After trying to model these results it was apparent that they do not correlate with amounts of heterochromatin or with sex chromosome combination or with an interaction of both.

The clear-cut difference in type of male sterility was first encountered by Craddock (1975) in the *planitibia* subgroup. Her data and unpublished data collected by Kaneshiro and by Ahearn reveal that females of *D. planitibia* crossed to any related species yield F₁ males with type I sterility whereas the reciprocal gives F₁ males of type II sterility. However, as Chang and Carson (1985) have shown, the only heterochromatic difference is between *D. hemipeza* and the others (*D. differens*, *D. planitibia*, *D. silvestris*, and *D. heteroneura*). *Drosophila hemipeza* has less heterochromatin in both the X and the largest autosome and probably more heterochromatin in the dot chromosome. The only chromosome that was not studied sufficiently to make conclusions was the Y. From genetic analyses of male fertility in *D. melanogaster* we know that seven fertility factors exist on the Y chromosome. In addition, genetic loci on autosomes and the relationship of centromere and chromosome tips are all critical and necessary to successful sperm development (Williamson 1976; Lindsley and Tokuyasu 1980; Gatti and Pimpinelli 1983). Furthermore, it has been shown that there are genetic elements in the heterochromatin of the X chromosome and the autosome(s) of *D. melanogaster* that may be associated with fertility factors on the Y chromosome (see Hilliker *et al.* 1980 for a review).

Drosophila bostrycha, *D. affinidisjuncta*, and *D. disjuncta* are outstanding examples of what we have yet to understand about speciation and species divergence. They have undergone karyotypic differentiation through addition and loss of heterochromatin. We know nothing about the mechanism. Carson (1981) states that heterochromatin alterations do not seem to be important in the dynamics of speciation. Certainly such alterations are the single major karyotypic difference between these three but whether this was the initial genetic change we don't know. Yoon and Richardson (1978) are of the opinion that heterochromatic changes lead to phenotypic changes. If there are phenotype differences between *disjuncta* and *affinidisjuncta* they certainly have nothing to do with morphology. Inter-specific hybrid male sterility is evidence for genetic differentiation but the genetic basis for the sterility is not understood. Polytenic band sequences, which reflect gene order arrangements, are shared in common by these three. Only *D. disjuncta* is polymorphic for unique inversions. Such data indicate that the species are related and are diverging but not how. Because they are homosequential with *D. grimshawi* and resemble it morphologically, a *D. grimshawi* like population was most probably the ancestor, but no simple scheme of progressive speciation events correlated with the age of the three volcanoes can be invoked.

There is strong evidence that changes in the genetic basis of courtship behaviour through sexual selection is the primary speciation event for Hawaiian picture wings (Carson 1978). Unfortunately, we have no information on courtship behavior for *bostrycha*, *disjuncta*, and *affinidisjuncta*. Another approach that has been valuable in identifying species relationships is restriction endonuclease cleavage site maps of mitochondrial DNA (Templeton 1983). We suggest such information would be useful in our future work as well.

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