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ETHOLOGICAL ISOLATION AND PHYLOGENY IN THE GRIMSHAWI SPECIES COMPLEX OF HAWAIIAN *DROSOPHILA*[1]

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The ethological isolation among six allopatric populations, comprised of two closely related species of Hawaiian *Drosophila*, was measured and used to analyze the phylogenetic relationships among these populations. The two species used in this study are included in the "picture-winged" species group (Carson and Stalker, 1968). In its present taxonomic status, *Drosophila grimshawi* Oldenberg is the only species of this group that inhabits all the accessible major islands (i.e. Kauai, Oahu, Maui, Lanai, and Molokai) except Hawaii. On the island of Hawaii a very closely related species, *D. pullipes* Hardy and Kaneshiro, is found. The only distinguishing morphological character between *grimshawi* and *pullipes* is reported to be the coloration of the legs and pleurae (Hardy and Kaneshiro, 1972).

A cytological comparison of the six populations showed a strong similarity between them. Carson and Stalker (1968) and Carson and Sato (1969) found *grimshawi* from the islands of Lanai, Maui, and Molokai (these islands known collectively as the "Maui complex of islands") to possess the standard polytene chromosomal sequence as well as one polymorphic inversion on the fourth chromosome (4A). Later studies (Carson et al., 1970) of *grimshawi* populations from Oahu and Kauai and of *pullipes* populations showed that they are fixed for the standard chromosomal sequence. That is, they differ from the Maui complex populations only in lacking the polymorphic 4A inversion sequence. For the *grimshawi* populations the comparison of metaphase karyotypes showed the five rods and one dot haploid chromosomal complement characteristic of most of the "picture-winged" species (Clayton, 1971). Therefore, contrary to the findings of Craddock (1974), no consistent morphological or cytological differences were detected among the insular populations of *grimshawi.*

Montgomery (1975) reared the adults of both species from field collected substrates and discovered an interesting ecological differentiation. The adults of the Maui complex *grimshawi* were obtained from the substrates representing 12 different plant families. However, *grimshawi* from Oahu and Kauai and *pullipes* from the island of Hawaii were reared exclusively from the rotting bark of trees of the genus *Wikstroemia* (family Thymeleaceae). On the basis of these studies *grimshawi* from the Maui complex have been termed ovipositional "generalists" whereas *pullipes* from Hawaii and *grimshawi* from Oahu and Kauai are ovipositional "specialists."

In addition to this ecological differentiation, Ringo (1974; 1976) observed jousting behavior differences among the males of some of these populations. Using stocks of Maui, Molokai, and Oahu *grimshawi* and *pullipies*, Ringo observed a greater difference in jousting displays between generalist and specialist males than between species. That is, although Maui and Molokai *grimshawi* had similar jousting displays as did Oahu *grimshawi* and *pul-*

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lipes, differences were apparent between these generalist and specialist males.

The ecological and ethological differentiation amidst virtual cytological and morphological identity prompted the study of the evolutionary relationships among the six populations. This study deals with male choice experiments between six stocks representing these allopatric populations. It will be shown here that considerable differences exist between reciprocal male choice experiments and that these data may provide a means by which a phylogenetic scheme may be constructed for the evolution of the populations studied.

MATERIALS AND METHODS

Six stocks were used for these experiments, each representing one of the six allopatric populations. Each of three of the *grimshawi* stocks, G1 (Maui), T59T20 (Lanai), and R83B13 (Molokai), were derived from a single, field collected, fertilized female. The Oahu *grimshawi* (R87Gα) and *pullipes* (R13Q) stocks were obtained from single pairs of adults reared from rotting *Wikstroemia* bark, and the Kauai *grimshawi* stock (T49T120) was derived from the collective larvae of 20 field collected females.

The methods used in the male choice experiments are described by Ahearn et al. (1974) and Kaneshiro (1976). A mature, virgin female from each of the two stocks to be tested was placed in a large 44 ml (32 mm × 98 mm) shell vial containing 4 ml of medium. A male from one of the two stocks was then immediately added to the vial, and the "trio" was maintained until the flies were dissected.

In the present study it was necessary to mark the females due to the identical morphology found among the populations of *grimshawi*. Marking was accomplished first by placing females of *grimshawi* and *pullipes* in a freezer at 0 C for five to 10 minutes until they were completely immobilized, then the flies were removed from the freezer and a small dab of Testor's (The Testor Corp., Rockford, Ill. 61101) fast-drying enamel paint was applied to the scutellar region of the thorax. The flies were allowed to recover for 10 to 15 min after which they were transferred and maintained for four to seven days. This extended duration between the treatment and experiment appeared to neutralize the short-term decrease in sexual activity of cold treated flies reported by Ringo (1971). In addition, all females were treated and marked (a different color for each of the six islands) in the same manner.

After establishment of the vials with the trios, a dark cloth was spread over the trays containing these vials, because *grimshawi* does not court or copulate in the dark (Grossfield, 1968; Ringo, 1977). The cloth was removed and the trios allowed to engage in courtship from approximately the hours of 0700 to 1000 and 1600 to 1800. Field experience has indicated that these were the hours during which maximum activity could be observed. A total of up to 60 vials were under constant observation during these periods to catch trios after one, but before the second, female was inseminated. When a copulation was observed the female that was not involved in copulation was immediately killed. The copulating pair was killed a few minutes after the completion of copulation and dissected immediately along with the female killed earlier. If the female that had not copulated had sperm in the spermathecae upon dissection and the female that was observed copulating had sperm only in the vaginal tract, it was assumed that the former was inseminated first. Although this situation occurred two or three times, this did not necessarily mean that the first copulation took place in the dark, because a few copulations may have gone unnoticed during the time taken for dissections. After three days of observations for those vials in which no mating had been observed males were allowed to court for the entire fourth day without observation. These trios were dissected at the end of the day and if both females were found to be virgins, the males were dissected to check for motile sperm and the data for those trios which had sterile males were discarded.

Trios were set up until at least 30 trials per experiment were successful (i.e. had only one female inseminated). Vials in which one female was inseminated only were used in the calculation of the Charles-Stalker isolation index (Stalker, 1942). The rationale for the elimination of vials in which both females were inseminated and those in which both females were virgin, from the calculation of the isolation index, is explained by Kaneshiro (1976).

The isolation index (I) is equal to the difference in percentages of heterogamic matings from homogamic matings divided by the sum of the percentages of homogamic and heterogamic matings (i.e. 100%). The isolation index has a maximum value of +1, meaning total isolation or all homogamic matings, and -1, meaning all heterogamic matings. A value of zero indicates that no isolation exists and that the male is equally acceptable to both females.

A test of proportions (C) was calculated for each I value which tests the null hypothesis that the two female types were mating at random ($I = 0$). The C value was calculated by using the formula $C = 2\sqrt{n}(p - 0.5)$ (Woolf, 1968) where: n = number of successful trios (i.e. only one female inseminated) and p = percentage of homogamic matings. The null hypothesis is accepted at the 5% confidence level if $-1.96 < C < +1.96$.

RESULTS

The behavioral isolation observed among the six stocks of *grimshawi* and *D. pullipes* is reported in Table 1. The first letter on the left of the table represents the stock (island) from which the male of the trio was obtained, and the second and third letters represent the stocks from which the two females were taken. The number of vials in which both females remained virgin were not used in the calculation of the isolation indices, but these data are nonetheless included in Table 1 since the number of vials with two virgins were used to calculate the receptivity of the females involved (Table 2). From Table 2 we can see that Kauai, Lanai, and Oahu females appear to be the least receptive, whereas Maui and Molokai females are the most receptive. For this reason Kauai and Lanai females were used as controls to test for the effects of chilling.

The test of the Kauai stock resulted in 11 successful trios out of 30. Of these 11, five had only the marked female and six only the unmarked female inseminated. The Lanai stock produced 21 successful trios out of 32. In 11 of these 21, only the marked female was inseminated, and in the remaining 10 only the unmarked female was inseminated. Therefore, it appears that the chilling and marking of the flies had no lasting effects on the courtship behavior of the females.

The important pattern to emerge from Table 1 was the "asymmetrical isolation" which was observed in nearly every reciprocal pair. This term refers to the significantly positive isolation observed with one set of trios coupled with the nonsignificant or significantly negative isolation obtained in the reciprocal.

The best example of asymmetrical isolation resulted from the testing of the Kauai stock against all others. Kauai males placed in vials with a Kauai female and a female from each of the other five stocks resulted in either a significantly negative isolation (e.g. with Oahu, Molokai, and Maui females) indicating more heterogamic than homogamic matings or no isolation (e.g. with Lanai females) indicating random mating. However, in the reciprocal direction (e.g. with Oahu, Molokai, Maui, and Lanai males) significant positive isolation resulted, evidenced by the increased number of homogamic matings.

The Oahu stock showed asymmetrical isolation similar to that of Kauai when it was

TABLE 1. *Male choice experiments between six populations consisting of two species:* D. grimshawi *and* D. pullipes. *(K = Kauai, O = Oahu, H = Hawaii, L = Lanai, R = Molokai, M = Maui).*

♂	♀♀	Vials with no matings	Vials with 2 matings	Only homogamic matings	Only heterogamic matings	I†	C*
K	KO	12	2	2	29	-0.87	-4.85
O	KO	7	2	26	4	0.73	4.02
K	KH	26	4	11	20	-0.29	-1.67
H	KH	10	0	30	0	1.00	5.48
K	KL	29	2	13	17	-0.13	-0.73
L	KL	9	0	32	0	1.00	5.66
K	KR	2	2	1	31	-0.94	-5.30
R	KR	8	0	31	0	1.00	5.57
K	KM	19	3	3	28	-0.81	-4.49
M	KM	25	0	33	0	1.00	5.74
O	OH	4	3	24	13	0.30	1.81
H	OH	3	2	31	1	0.94	5.30
O	OL	13	2	24	8	0.50	2.83
L	LO	35	0	24	7	0.55	3.05
O	OR	8	2	14	23	-0.24	-1.48
R	OR	1	0	30	2	0.87	4.95
O	OM	1	4	6	26	-0.63	-3.53
M	OM	17	0	30	0	1.00	5.48
L	LH	22	3	18	13	0.16	0.90
H	LH	2	6	30	5	0.71	4.23
L	LR	10	0	3	28	-0.81	-4.49
R	LR	1	1	23	8	0.48	2.69
L	LM	12	5	15	19	-0.12	-0.69
M	LM	11	3	29	11	0.45	2.85
H	HR	6	6	18	14	0.13	0.71
R	HR	2	0	31	1	0.94	5.30
H	HM	8	1	6	24	-0.60	-3.29
M	HM	10	0	29	1	0.93	5.11
R	RM	0	5	16	17	-0.03	-0.17
M	RM	10	8	12	26	-0.37	-2.27

† I = Charles-Stalker isolation index (see Methods section).

* C = Test of proportions. The null hypothesis that mating is random is accepted if $-1.96 < C < +1.96$ (see Methods section).

tested against the Maui and Molokai stocks. Thus, Oahu males copulated with Maui and Molokai females very readily, while Maui and Molokai males found it difficult to copulate with Oahu females. When the Oahu stock was tested against that of Lanai, however, isolation was apparent in both reciprocal combinations and a "reciprocal isolation" was obtained. In the Oahu-Lanai combination the isolation indices were similarly positive, both reciprocals showing isolation. In the Oahu-Hawaii combination there was a tendency for asymmetrical isolation as Hawaii males found it more difficult to copulate with Oahu females than Oahu males did with Hawaii females. In fact, when Oahu males were used, mating was random as the C value was not significant; but its value, being very close to a significantly positive isolation, merits attention.

TABLE 2. *Female insemination rates and the hierarchy of female receptivity in male choice experiments between six populations of* D. grimshawi *and* D. pullipes. *(K = Kauai, O = Oahu, H = Hawaii, L = Lanai, R = Molokai, M = Maui).*

K	34/458 = 0.074	H	163/413 = 0.395
O	133/425 = 0.313	M	247/444 = 0.556
L	141/471 = 0.299	R	253/377 = 0.671

$$K \overset{**}{<} L = O \overset{*}{<} H \overset{**}{<} M \overset{*}{<} R$$

* $P < 0.05$, ** $P < 0.01$, using chi-square tests.

When the Lanai stock was tested against Hawaii, Molokai, and Maui stocks each combination showed asymmetrical isolation. The Lanai males mated with Hawaii, Molokai, and Maui females without difficulty but their males had some difficulty mating with the Lanai females. The Hawaii stock showed similar results when tested against those of Maui and Molokai, as Hawaii males found no difficulty in copulating with Maui and Molokai females but their males had greater difficulty in copulating with Hawaii females.

The Maui-Molokai combination showed no asymmetrical isolation, and females of both stocks randomly accepted intrastock as well as interstock males. This lack of isolation between these two stocks was not unexpected in light of the high receptivity of the females of both of these stocks (Table 2).

DISCUSSION

The complexity of *Drosophila* courtship patterns (Spieth, 1952; 1966*a*; 1966*b*) indicates the polygenic nature of this behavior. Ehrman (1961) found sexual isolation in *D. paulistorum* under the control of polygenes on each of its three pairs of chromosomes. The polygenic nature of sexual isolation is the probable cause of its high susceptibility to genetic change. Even single gene mutations in *D. melanogaster* have been shown to affect courtship patterns (Rendel, 1951; Reed and Reed, 1950; Bastock, 1956).

Kaneshiro (1976) theorized that the asymmetrical isolation observed in the planitibia species group may be due to a change in courtship behavior patterns in response to a genetic revolution brought about by the founder effect (Carson, 1968; 1971). Using the geological age of the island of Hawaii to indicate that two species present on this island are derived from species found on Maui and Molokai, he proceeded to show that females of such derived species tend to accept the courtship overtures of males faster than females of ancestral species and speculated that this may be due to a loss of courtship elements (either qualitative or quantitative in nature) during the founder event.

Populations formed from single founders may lose courtship elements due to selection for less discriminatory females. When a founder establishes a new population the population size must be extremely small. The chance meeting of conspecific males and females will then be relatively rare, and those females which are very discriminating have a lesser chance of being inseminated than those which are very receptive. Therefore, selection may favor less discriminating females and operate until the population builds to a reasonable size. As the size and range of the population increases, other species will be encountered and some of these may possess courtship patterns very similar to the founders. Thus, new courtship elements may arise in the new population to prevent hybridization.

If we accept the above explanation for the occurrence of asymmetrical isolation, then it is possible to predict the phylogenetic sequence of events for these six populations using the data in Table 1. The data appear to indicate that Kauai *grimshawi* is ancestral to all other *grimshawi* populations and *pullipes*. Kauai males were acceptable to all females of the other populations (Oahu, Maui, Lanai, Molokai, and Hawaii). This is reflected in the negative *I* values whenever Kauai males were used. On the other hand, Kauai females rejected the males from all other populations as indicated by the significantly positive isolation indices. Therefore, all populations except Kauai may have lost courtship elements resulting in the observed asymmetrical isolation.

Similarly, the Oahu population appears to be ancestral to those from Maui and Molokai. The significantly positive isolation observed in both reciprocals, when Oahu and Lanai populations were tested may have been the result of two separate founders from Kauai establishing the Oahu and Lanai populations. If Oahu and Lanai populations were from separate founders, it is more than likely that different sets of courtship elements would be lost due to drift. This would lead to isolation in both reciprocals as each population would require some elements which the other lacked. Another explanation for reciprocal isolation is that some elements may have changed or new elements were added in either the Oahu or the Lanai populations, resulting in courtship elements unique to that population. However, if this were true, the Kauai stock should also have shown reciprocal isolation with that stock.

The experiments involving Oahu and Hawaii stocks show what may be partial isolation with Oahu males and nearly total isolation with Hawaii males. If the Hawaii population was derived from the Oahu population, random mating or a negative isolation index is expected with Oahu males. This is indeed the case as the *C* value is not significant and therefore, random mating is indicated. However, the near significance of the *C* value strongly suggests that some isolation may exist between these populations. The partial isolation of Hawaii males from Oahu females may indicate that the Hawaii population was derived from a Lanai ancestor and during the founder event some of the same elements which the Oahu population lacks, but are present in the Lanai population, were lost. The asymmetrical isolation observed when the Lanai and Hawaii stocks were tested provides further evidence that the Hawaii population was founded from Lanai.

The Lanai stock when tested against Maui and Molokai stocks appears to be ancestral to both of them. Likewise, the Hawaii population appears to be ancestral to both Maui and Molokai populations. However, the Maui and Molokai stocks when compared against one another appear to mate randomly in both reciprocal combinations, indicating that these populations may share similar complements of courtship elements. This lack of isolation between Maui and Molokai stocks may have been caused by the absence of a founder effect in the establishment of one of these populations from the other. If the establishment of the Maui or Molokai population from the Molokai or Maui population respectively, occurred during the late Pleistocene, migration of several flies may have been possible as during this geological period, the now separate islands of the Maui complex were probably connected by land bridges due to a drop in the sea level (Stearns, 1966). Therefore, in the absence of the founder event very few elements, if any, were lost.

The evolutionary sequence of events, as indicated by the observed isolation indices, seem to have begun with the Kauai population. This is consistent with the geological age of Kauai, which is the oldest of the high islands. From Kauai two separate founders established populations on Oahu and Lanai and from Lanai, a Hawaii population was derived. At this point contradictory evidence exists for the establishment of the Maui-Molokai populations. Behaviorally (using the theory of Kaneshiro, 1976) the Hawaii population founded the Maui-Molokai populations, but ecologically and geologically (age of the islands) the Maui-Molokai populations arose from a Lanai founder (Lanai, Maui, and Molokai *grimshawi* are all generalists, but *pullipes* is a specialist). The latter scheme appears to be the more acceptable one, since with this scheme (Fig. 1), the evolution of generalism need only to occur once, namely on Lanai. If a Lanai individual founded the Hawaii population prior to the evolution of generalism but founded the Maui-Molokai populations subsequent to it, then the generalism event in *grimshawi* need only to have occurred once.

The asymmetrical isolation found in the comparison of the Maui-Molokai populations with all others including Hawaii may be explained by a large loss of elements when the Maui or Molokai population was founded. This loss of elements may have

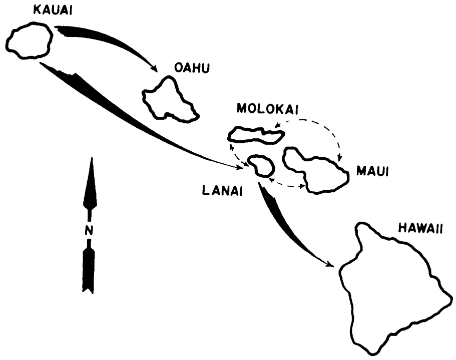

FIG. 1. Proposed phylogeny for six populations in the grimshawi complex of species using behavioral data.

included those elements which Lanai, Oahu, and Hawaii populations lacked as well as some additional elements. This loss of elements in these populations may account, at least in part, for the high receptivity of the females from the stocks representing these islands as they may require less courtship elements before acceptance of the male.

SUMMARY

The theory that asymmetrical isolation reflects phylogenetic origins (Kaneshiro, 1976) is generally supported by this study. All of the phenomena observed were explainable using this theory, although interpretation of some of the data required the use of the available ecological data. Although, as yet, no direct evidence for the loss of courtship elements has been provided, the consistency of the asymmetrical isolation observed in most reciprocal combinations and the agreement of the phylogeny with the geological age of the islands suggests that this may be a reasonable explanation for the observed phenomenon. The Kaneshiro theory of asymmetrical isolation may be a highly significant contribution to evolutionary theory because progressive evolution arrows may be drawn in one direction only.

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