

EVOLUTIONARY RELATIONSHIPS OF FOUR SPECIES OF HAWAIIAN DROSOPHILA AS MEASURED BY DNA REASSOCIATION

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ABSTRACT

Four species of the Hawaiian *Drosophila planitibia* subgroup which are homosequential in their polytene chromosomes are resident on the islands of Molokai, Maui and Hawaii. Comparisons of DNA sequence divergence in these four have been made by hybridization of total single-copy radiolabeled tracer DNA from each of the species with excess nonlabeled DNA from each of the species, and measurement of the reduction of average melting temperature (ΔT_{ma}) was made in 2.4 M tetraethyl ammonium chloride. The mean ΔT_{ma} between either *D. heteroneura* or *D. silvestris* and either *D. planitibia* or *D. differens* was found to be 1.06°, whereas the difference between *D. planitibia* and *D. differens* is 0.65° and between *D. heteroneura* and *D. silvestris* is 0.75°. These measurements taken together with the distances calculated from isozyme studies, chromosomal relationships, as well as the island locations indicate that the ancestor of these species diverged from other *planitibia* subgroup flies on Molokai [age 1.8 million years before present, (My BP)]. We hypothesize that one line became the present-day *D. differens* and diverged probably at the time of formation of East Maui (0.8–1 My BP) to form the species *D. planitibia*. Flies from the other line migrated to Hawaii soon after its formation (0.7 My BP) to form the two species *D. heteroneura* and *D. silvestris*.

THE four species of Hawaiian *Drosophila*, *D. heteroneura*, *D. silvestris*, *D. planitibia* and *D. differens*, are close relatives from the picture-wing group of *Drosophila* species found in the high altitude rainforests of the newer Hawaiian Islands. The first two species are endemic to the island of Hawaii, where they are broadly sympatric. *D. planitibia* and *D. differens* are endemic to Maui and Molokai, respectively. Their evolutionary relationships have been studied using morphological characteristics (CARSON and KANESHIRO 1976), polytene chromosome banding homology (CARSON and YOON 1982), isozyme studies (SENE and CARSON 1977; CRADDOCK and JOHNSON 1979) and ethological isolation and behavior (KANESHIRO 1976; SPIETH 1981). The speciation process can also be related to the time of formation of the islands upon which they are found (CARSON and YOON 1982).

Of all of these measurements, except possibly for age of the island of origin, the only one that gives a numerical measure of evolutionary distance is the

isozyme analysis. However, for the four species in question analysis has been limited to gene frequencies of 12 enzyme systems between *D. differens* (D) and *D. planitibia* (P) and 24 enzyme systems between *D. heteroneura* (H) and *D. silvestris* (S). A distance (\bar{D} , NEI 1975) of 0.23 is found between the D-P pair and the H-S pair. Within each pair \bar{D} is 0.11 for *D. planitibia* and *D. differens* and 0.02–0.08 for *D. heteroneura* and *D. silvestris* (SENE and CARSON 1977; CRADDOCK and JOHNSON 1979). Chromosomally, *D. heteroneura* and *D. silvestris* are homosequential in polytene chromosomes with *D. planitibia* and *D. differens*. The former pair, however, shares a polymorphic inversion (CARSON and KANESHIRO 1976).

We have now measured the distances between these species by DNA reassociation of single-copy tracer DNA from each of the species and by measurement of the lowering of melting temperature of the hybrids. We conclude that the two pairs of species (D-P and H-S) diverged from each other at essentially the same time, and each pair derived from a common ancestral species that had colonized and diverged on the island on Molokai.

METHODS

Drosophila stocks were maintained from wild-caught animals. The *D. heteroneura* stock was from an isofemale line from the Olaa Tract, Hawaii Volcanoes National Park on the island of Hawaii in 1972 (Q71G12). The *D. silvestris* stock was from an isofemale line from the Kilauea Forest Reserve on the island of Hawaii established in 1977 (U28T2). The *D. differens* stock was from an isofemale line from Hanalilolilo on the island of Molokai established in 1979 (U43V1), and *D. planitibia* was from a massed F_1 generation reared from a branch of *Clermontia* from Waikamoi on the island of Maui in 1979 (U84Y).

DNA extraction: All DNA was extracted from 3rd instar larvae by homogenization in 6 M guanidium chloride and centrifugation in cesium chloride as previously described (HUNT, HALL and BRITTEN 1981).

Preparation of ^3H -labeled DNA: Nick translation of acid-activated deoxyribonuclease-treated DNA samples (5 ng/ μg of DNA) using *E. coli* DNA polymerase from Bethesda Research Laboratories and (^3H -methyl) TTP (specific activity 67.3 Ci/mmol) was by the method of RIGBY *et al.* (1977). The labeled DNA was denatured and reannealed to an equivalent Cot of 10 mol sec liter $^{-1}$ in 0.5 M sodium phosphate, pH 7.0, and snap back and repetitive DNA was removed by chromatography on hydroxyapatite (BRITTEN, GRAHAM and NEUFELD 1974).

Annealing of DNA with radiolabeled DNA: These methods have been described previously (HUNT, HALL and BRITTEN 1981). Sheared driver DNA of size 800–1000 nucleotides in length was reassociated in 1.0 M tetraethyl-ammonium chloride (TEACL) at 45° with ^3H -labeled single-copy DNA, using a 1000-fold excess of driver DNA (20,000 cpm of ^3H -labeled DNA and 20 μg of driver DNA), and the reaction was terminated at an equivalent Cot of greater than 6000 mol sec liter $^{-1}$. Digestion to 90–95% completion was accomplished with these samples by dilution with an equal volume of water and two volumes of 0.2 M Na acetate, 0.002 M ZnCl_2 , pH 4.4 (total volume 50 μl) using 1.5 units of nuclease S_1 (Bethesda Research Laboratories) for 20 μg of DNA at 37° for 1 hr. The reaction was terminated by adding EDTA to 3×10^{-2} M, and a 10- μl aliquot was saved for size analysis. The buffer was changed to a 2.4 M TEACL by chromatography on Sephadex G100 for melting point determination.

Melting point determination: Melting point determinations in a heating block were determined as described previously (HUNT, HALL and BRITTEN 1981). Nuclease S_1 digestion was in a final concentration of 0.1 M Na acetate, 0.001 M ZnCl_2 , pH 4.4, and 6 μg of heat-denatured sheared calf thymus DNA was added to ensure complete digestion by the nuclease S_1 (25 units/reaction). The undigested DNA was precipitated with 2% cetyltrimethyl ammonium bromide, and both the precipitate and supernatant were assayed for radioactivity. The scintillation cocktail used was 10 ml of Biofluor (New England Nuclear) per 1 ml of assay solution.

Size analysis of DNA fragments: The nuclease S_1 -treated reassociated DNA was sized on horizontal gel electrophoresis in 3% alkaline agarose gels exactly as described previously (HUNT, HALL and BRITTEN 1981).

Calculating of melting temperature: The average melting temperature (T_{ma}) is used in these determinations and is obtained by calculating the area under the melting curve (HUNT, HALL and BRITTEN 1981). This enables an accurate correction to be made from the size analysis of the tracer DNA after the first nuclease S_1 digestion.

RESULTS

DNA preparations: The size distribution of the tracer DNAs used in these experiments was determined by alkaline agarose gel electrophoresis. The number average size is 420–530 nucleotides and the weight average size 890–1100 nucleotides. All of the DNA preparations had a specific activity of greater than 1.5×10^6 cpm/ μ g. The median size of the sheared DNA samples was estimated as approximately 1000 nucleotides by alkaline agarose electrophoresis.

DNA hybridization: Each of the four tracer DNA samples was hybridized in duplicate with the four samples of DNA from the same preparations used to synthesize the tracers. Melting curves were performed on each of the 16 duplicate hybridizations. Melting curves from one set of hybridizations are shown in Figure 1. The T_{ma} is determined by integrating the curve from 35° to above 65° . These values are shown in Table 1. The correction factors due to the size of the tracer DNA after the initial nuclease S_1 digestion are calculated from the alkaline agarose gel electrophoresis and are shown as either the number

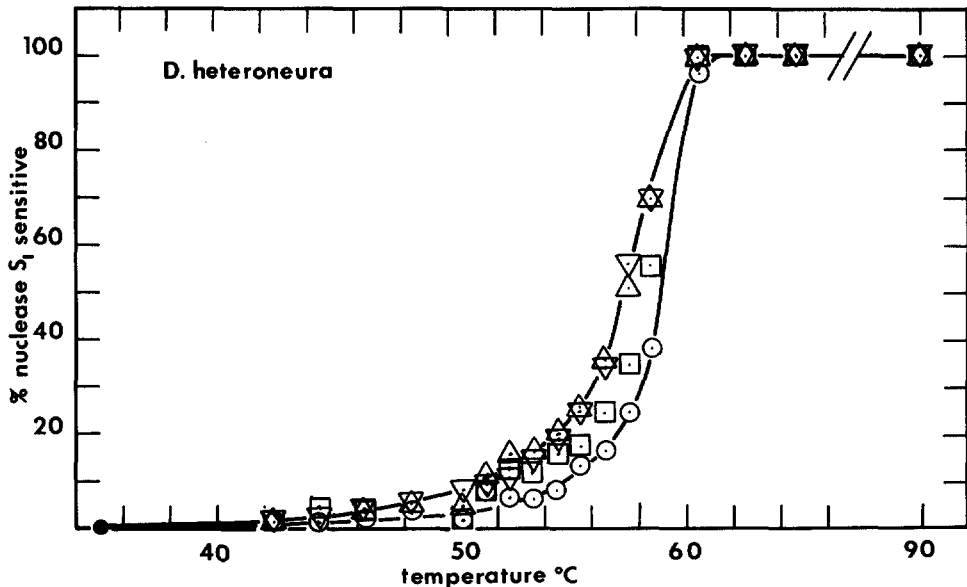


FIGURE 1.—Examples of melting curves of the hybrid DNAs from the four species. Each curve is normalized from 0–100%. Driver DNA species are represented by the following symbols: ○, *D. heteroneura*; □, *D. silvestris*; △, *D. planitibia*; ▽, *D. differens*. The tracer DNA was from *D. heteroneura*. The initial and final percentage of nuclease S_1 -digestible DNA was for *D. heteroneura*, 5.2 and 95.3; *D. silvestris*, 5.2 and 95.8; *D. planitibia*, 5.2 and 95.6; and *D. differens*, 5.2 and 96.8.

TABLE 1
Hybridization of the four species of DNA

Driver DNA	<i>D. heteroneura</i>				<i>D. silvestris</i>				<i>D. planitibia</i>				<i>D. differens</i>				
	H	S	P	D	H	S	P	D	H	S	P	D	H	S	P	D	
T _{ma} (°) ^a																	
1	58.11	57.27	56.46	56.42	57.09	57.94	56.66	56.79	56.51	56.70	57.56	56.99	55.74	55.28	55.85	56.68	
2	57.78	56.76	56.15	56.10	57.49	58.26	56.68	56.61	57.27	56.37	57.16	56.83	55.33	54.97	55.53	56.47	
Tracer length (nucleotides) ^b																	
1	288	265	217	242	254	275	233	237	222	299	266	265	188	169	174	181	
2	236	225	207	199	213	223	193	195	186	192	198	217	168	155	166	177	
T _{ma} correction (°) ^c																	
1	1.74	1.88	2.30	2.07	1.97	1.82	2.15	2.11	2.25	1.67	1.88	1.89	2.66	2.96	2.87	2.76	
2	2.12	2.22	2.41	2.52	2.34	2.24	2.59	2.56	2.69	2.61	2.52	2.30	2.98	3.23	3.00	2.83	
% reaction ^d																	
1	74.4	76.0	72.1	70.6	70.2	76.2	69.1	65.7	65.4	56.1	74.6	63.4	53.6	53.5	57.3	55.2	
2	70.0	70.7	68.2	66.7	66.8	66.5	62.3	62.4	61.6	61.4	64.4	62.2	43.1	45.3	45.5	45.8	
Normalized % reaction ^e																	
1	103	97	97	96	92	89	89	91	89	76	86	86	88	87	92	92	
2	100	97	97	97	101	94	94	96	95	95	97	97	90	93	89	89	
ΔT _{ma} ^f																	
1	0.84	1.69	1.63	1.65	0.85	1.28	1.28	1.15	1.05	0.86	0.57	0.57	0.94	1.40	0.83	0.83	
2	1.02	1.63	1.63	1.68	0.77	1.58	1.58	1.65	0.89	0.79	0.33	0.33	1.14	1.50	0.94	0.94	
ΔT _{ma} corrected ^g																	
1	0.70	1.09	1.09	1.36	0.70	0.95	0.95	0.86	0.68	1.07	0.56	0.56	1.04	1.20	0.72	0.72	
2	0.92	1.34	1.34	1.28	0.67	1.23	1.23	1.33	0.72	0.70	0.55	0.55	0.99	1.10	0.77	0.77	
Mean	0.81	1.21	1.21	1.32	0.69	1.09	1.09	1.10	0.70	0.89	0.56	0.56	1.02	1.15	0.75	0.75	

^a 1 and 2 refer to experiments 1 and 2.

^b The tracer length is the number average length $\Sigma Q_i / \Sigma(Q_i/L_i)$ when Q is the number of counts in each slice of the agarose gel and L_i the average length of DNA in that slice.

^c The T_{ma} correction is $500 \Sigma(QE_i/L_i) / \Sigma Q_i$.

^d Percent reaction is the percentage of tracer DNA undigested at 95% nuclease S₁ digestion.

^e The normalized reaction is the percent reaction corrected for the fraction of the duplex digested by the 99% nuclease S₁ reaction for each tracer relative to the hybridization with the homologous driver DNA.

^f Difference of the T_{ma} between the homologous and heterologous hybridizations.

^g Difference of T_{ma} plus T_{ma} correction between the homologous and heterologous hybridizations.

average size in nucleotides or as ΔT_{ma} correction which is taken as 500°/number average size in nucleotides (HALL, HALL and BRITTEN 1980).

Analysis of the corrected ΔT_{ma} values by the Wilcoxon rank order test for the postulate that the within-pair H vs. S or D vs. P values are lower than the between-pair values (H or S vs. D or P) was significant at the 0.1% level. There was no significant difference in ranks when each of the H or S vs. D or P values was tested against the others of this group using the same test.

The values obtained using the *D. planitibia* tracer are all lower than expected, although the between-pair values (P vs. S or H) are still higher than the within-pair values (P vs. D), and the measurements using the *D. planitibia* tracer produced the same rank order as for the other experiments. This could be explained if the *D. planitibia* stock, which was not from an isofemale line, had a greater degree of heterozygosity than the isofemale line stocks used for the other species. In this case we would predict a greater depression of the homologous melting point with a corresponding increase in the T_{ma} of the reciprocal melts which is found.

The summary of the ΔT_{ma} values found for reciprocal pairs of hybridization is shown in Table 2. By using the Kruskal Wallace rank order test to test for the hypothesis that each of the H or S vs. P or D values (lines 1-4) are homogeneous, we find a χ^2 (3 d.f.) of 1.1. This indicates that the four pairs of measurements are equivalent. When the H vs. S or P vs. D values are added, heterogeneity is found with a χ^2 (5 d.f.) of 11.7 ($> 0.05 > P > 0.025$).

DISCUSSION

Several schemes have been proposed to represent the evolutionary relationships of the four species discussed here. Using chromosome inversions and island location, CARSON and YOON (1982) infer two ancestral populations. The older of these is on Molokai. Two separate lines of descent gave rise to (1)

TABLE 2
Values of ΔT_{ma} for reciprocal pairs of hybridization

Species pair ^a	ΔT_{ma}	Species pair	ΔT_{ma}	Within pair mean ΔT_{ma}	S.D. ^b
H/D	1.32	D/H	1.02	1.17	± 0.18
S/D	1.09	D/S	1.15	1.12	± 0.20
S/P	1.09	P/S	0.88	0.99	± 0.22
H/P	1.21	P/H	0.70	0.96	± 0.31
Mean of ΔT_{ma} for species pair above				1.06	± 0.10
H/S	0.81	S/H	0.68	0.75	± 0.12
D/P	0.75	P/D	0.55	0.65	± 0.11

^a The first letter in H/D refers to the tracer DNA and the second to the driver DNA, i.e., *D. heteroneura* tracer DNA with *D. differens* driver DNA.

^b The standard deviation is derived from the duplicate experiments and the reciprocal hybridizations, except for the between-species mean where the standard deviation is derived from the mean values of each pairwise comparison.

modern *D. planitibia* on Maui and (2) modern *D. differens* on Molokai. The second ancestral population was derived from the first and gave rise to *D. heteroneura* and *D. silvestris* on the island of Hawaii after a single interisland colonization. This scheme may be visualized by deleting the arm marked "A" in Figure 5.

A different scenario has been proposed by KANESHIRO (1976). Based on morphological characters (see CARSON and KANESHIRO 1976) and two different kinds of behavioral data, it also infers two ancestral populations for these species (see Figure 2). One is on Molokai and the other on Maui. The latter serves as a common ancestor for *D. planitibia* (Maui) and *D. silvestris* (Hawaii). *D. heteroneura* of Hawaii is postulated to have arisen separately either from the Molokai or the Maui ancestor.

The conventional genetic distance found by measuring frequencies of protein polymorphism and summarizing them in a single statistic, \bar{D} (NEI 1975), for the four species can be calculated from the data published by SENE and CARSON (1977) and by Craddock and JOHNSON (1979). The latter study was mostly concentrated on the comparison of *D. silvestris* from nine localities on the island of Hawaii. *D. heteroneura* from one location, *D. planitibia* from East and West Maui and *D. differens* from Molokai were also studied. Only 12 enzyme systems were studied, and the original similarities were measured using a different statistic (ROGERS 1972). These have been recalculated as \bar{D} and are shown in Table 3.

SENE and CARSON (1977) compared gene frequencies of 24 enzymes from *D. silvestris* and *D. heteroneura* from three locations on the island of Hawaii.

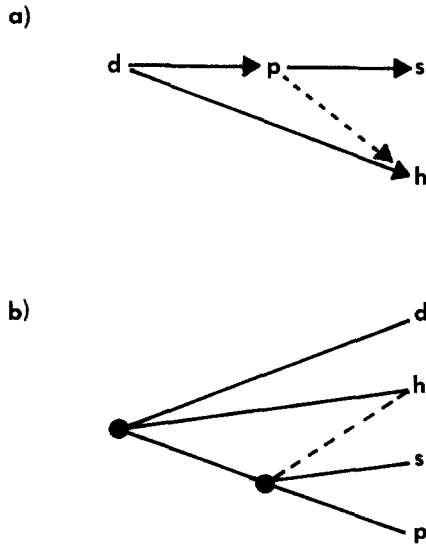


FIGURE 2.—a, Postulated phylogenetic relationships of *D. differens* (d), *D. planitibia* (p), *D. silvestris* (s) and *D. heteroneura* (h) as diagrammed by KANESHIRO (1976). b, The same, showing the two inferred ancestral populations (closed circles). The dotted line indicates an alternative pathway for *D. heteroneura*.

These data have been recalculated to include only those 12 enzymes studied by CRADDOCK and JOHNSON (1979) to facilitate comparison with the other two species. These data are shown in Table 4. With these values a dendrogram of the relationships between the four species was calculated by using the average distance between the species (FITCH and MARGOLIASH 1967), and this is shown in Figure 3.

The distances calculated in this work using DNA divergence can also be used to form a dendrogram of the relationships among the four species. This is shown in Figure 4. In this case we have averaged the values obtained by reciprocal hybridization, since this corrects for both differences in the DNA preparations and the degree of heterozygosity within each species (HUNT, HALL and BRITTEN 1981).

The KANESHIRO hypothesis (Figure 2) is based partly on morphology, partly on isolation indices and partly on mating asymmetry. If only the latter criterion is used, the data are not inconsistent with the other schemes, since *D. silvestris* could as easily have been derived from the Molokai ancestor as from the Maui ancestor. We feel that morphological similarities and similarity of isolation indices are outweighed by the quantitative data available from the DNA and protein similarity relationships.

TABLE 3

Genetic distance (\bar{D}), using 12 enzyme systems, between the species *D. heteroneura*, *D. silvestris*, *D. planitibia* and *D. differens* calculated from data of Craddock and Johnson (1979)

	\bar{D}			
	<i>D. heteroneura</i>	<i>D. planitibia</i>		<i>D. differens</i>
		East Maui	West Maui	
<i>D. silvestris</i>	0.021	0.272	0.228	0.262
<i>D. heteroneura</i>		0.194	0.233	0.229
<i>D. planitibia</i>				
East Maui			0.093	0.102
West Maui				0.124

TABLE 4

Genetic distance (\bar{D}) for the same 12 enzyme systems as in Table 3a for pairwise comparisons of *D. silvestris* and *D. heteroneura* from different locations on the island of Hawaii calculated from data of Sene and Carson (1977)

<i>D. silvestris</i> from	\bar{D}			
	<i>D. heteroneura</i> from			Mean
	Kahuku	Olaa	Pauahi	
Kahuku	0.047	0.044	0.167	0.084
Olaa	0.017	0.018	0.127	0.052
Pauahi	0.073	0.080	0.155	0.102
Mean	0.045	0.080	0.150	0.079

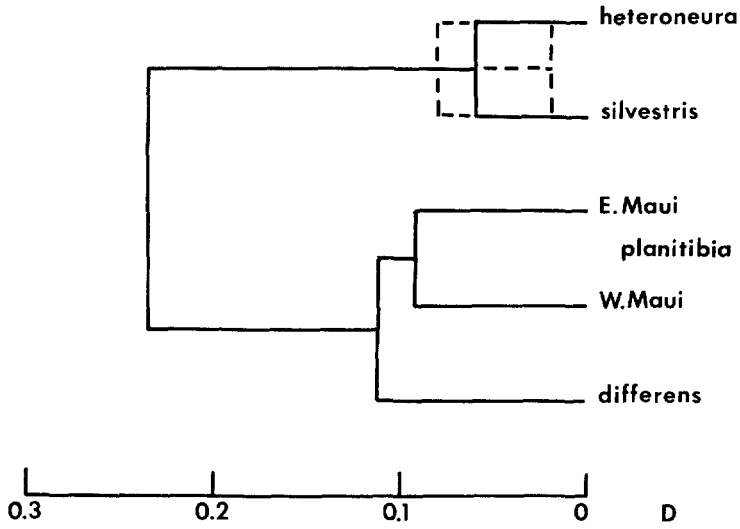


FIGURE 3.—The dendrogram of relationships of the four species as determined by isozyme distances (\bar{D}) taken from data of CRADDOCK and JOHNSON (1979) and SENE and CARSON (1977). The dotted lines represent maximum and minimum values from different populations.

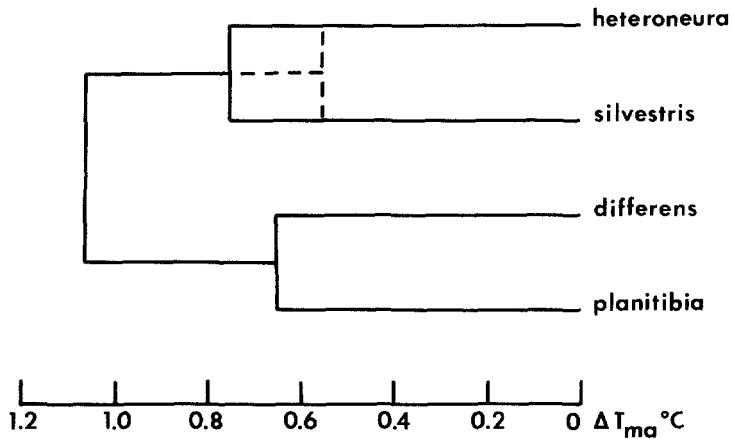


FIGURE 4.—The dendrogram of relationships of the four species as determined in this study using ΔT_{ma} by DNA hybridization. The dotted line incorporates previous data for *D. heteroneura* and *D. silvestris* made by the same method (HUNT, HALL and BRITTEN 1981).

There are differences between these quantitative measures, but they are in degree rather than in the absolute arrangement. One problem with the protein polymorphism studies from which the \bar{D} values were calculated is the small number of enzymes studied. The major comparison among the four species used only 12 enzymes, and the values for \bar{D} between *D. heteroneura* and *D. silvestris* in two studies using the same 12 loci are between 0.02 and 0.08, whereas the difference between *D. planitibia* and *D. differens* in the only study made is 0.11. The standard error for the latter measurement is 0.1 (NEI and ROYCHOUDHURY 1974). When SENE and CARSON (1977) extended the measure-

ments between *D. heteroneura* and *D. silvestris* to 24 loci \bar{D} became 0.06 ± 0.02 varying from 0.03–0.1 depending on the populations compared.

If we combine our present data with the previous measurements made with DNA from *D. heteroneura* and *D. silvestris* we find that the ΔT_{ma} between these species is $0.63 \pm 0.2^\circ$. These results illustrate that it is technically difficult to measure differences between such very closely related species accurately by measuring the lowering of melting point of DNA hybrids. We believe that the standard deviation of 0.2° found for the measurements from closely related species indicates that values of 0.5° are very close to the limit of accuracy. There would be little gained from making repeat measurements; this contrasts with the possibility of increasing the accuracy of \bar{D} from enzyme polymorphism studies using more enzymes rather than more individuals (NEI and ROYCHOUDHURY 1974).

There is no significant difference between the ΔT_{ma} found between *D. heteroneura* and *D. silvestris* and that found between *D. differens* and *D. planitibia*. Moreover, the large standard deviation in the \bar{D} value based on only 12 enzyme loci does not allow detection of a significant difference in the within-pair \bar{D} values for *D. heteroneura* and *D. silvestris* and *D. differens* and *D. planitibia*.

The dating for the divergence of species which occupy the Maui complex (Molokai, Maui, Lanai) may be complicated by the action of the ice age in the Pleistocene (CARSON and YOON 1982; MACDONALD and ABBOTT 1970). During that time the islands were joined into one land mass, and the sea level was lowered by up to 300 m. At the same time the cooler climate probably caused the mesic forests which are now at elevations above 600 m to cover the lower levels.

However, from consideration of the distances measured by both DNA hybridization and protein polymorphism it appears that each of the two sister species diverged at approximately the same time but that the divergence between the pairs occurred almost twice as long ago as the time of the within-pair divergence. The most probable scheme of evolutionary divergence of these four species is shown in Figure 5.

Our data deal with four cytologically very similar species of the *planitibia* subgroup, currently found on the Maui complex and Hawaii. Using cytological evidence, CARSON and YOON (1982, Figure 12) have suggested that there was a major burst of evolution in this subgroup on the island of Molokai (age 1.8 My BP, MACDONALD and ABBOTT 1970), ultimately producing 14 of the 17 currently existing species. The four species we discuss here are unique in having the Xr inversion in fixed condition. The DNA relationships among the four suggest that two ancestral Xr populations existed. One of these lines produced the present-day *differens-planitibia* species-pair; the latter was established as a new species by colonization of West and East Maui [age of Maui volcanoes are 1.3 and 0.8 My BP, respectively (MACDONALD and ABBOTT 1970)]. The other line acquired a polymorphic inversion (3m/+) either before or after colonization of Hawaii (age of island 0.7 My BP, McDOUGALL 1969) and gave rise to the *heteroneura-silvestris* pair. Both members of this species-pair retained this chromosomal polymorphism. The DNA distance values of 0.63, 0.65 and 1.1°

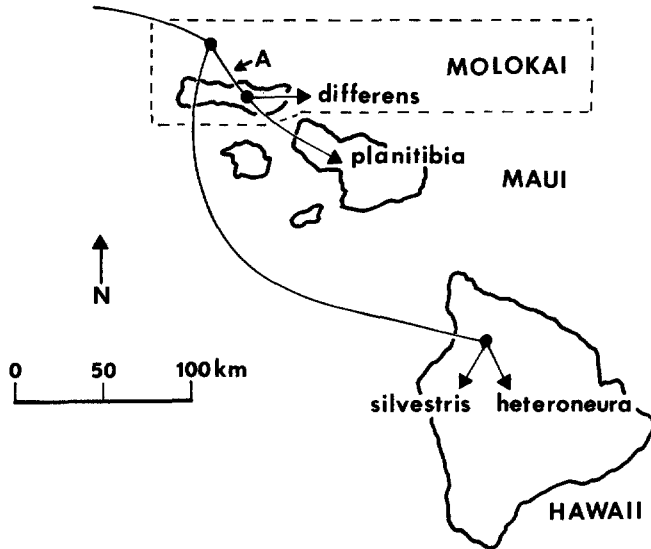


FIGURE 5.—A revision of the colonization relationships of the four species taking into account the interspecies differences measured by protein polymorphism \bar{D} and DNA ΔT_{ma} . The phylogeny proposed by CARSON and YOON (1982) can be visualized on this figure by deleting the line marked A. Closed circles represent ancestral populations from which the species evolved.

ΔT_{ma} found between lines (Figure 2) are consistent with the divergence times of 0.7, 0.8–1.3 and 1.8 My, respectively, being the earliest times that the Islands could have been available for habitation. The rate of divergence, assuming that 1° difference in T_{ma} is caused by a 1% sequence difference (HUNT, HALL and BRITTEN 1981), is 0.45, 0.27, and 0.31%/My, respectively, for the H vs. S; P vs. D; and H or S vs. P or D pairs using maximal times of divergence from island ages, namely, Hawaii 0.8 My, Maui 1.3 My and Molokai 1.8 My. An alternative to this would be that *D. heteroneura* and *D. silvestris* diverged before they colonized Hawaii. However, the lack of accuracy in both the protein polymorphism and DNA measurements does not allow us to distinguish between these models.

The difficulty of knowing when colonization and speciation occurred only allows estimates of maximal or minimal times of divergence. For example, if we consider *D. picticornis* from the island of Kauai, which has $2.1^\circ \Delta T_{ma}$ from either *E. silvestris* or *D. heteroneura* (HUNT, HALL and BRITTEN 1981), there are a large number of chromosome inversion differences between *D. picticornis* and the presumed colonizer of Oahu, the next island in the Hawaiian chain, which eventually lead up to *D. heteroneura* and *D. silvestris* (CARSON and YOON 1982). Thus, we would assume a minimum time of divergence of *D. picticornis* and the ancestor of the other species as the age of the island of Oahu (3.3 My). However, the divergence could have come as early as the colonization of the island of Kauai (5.6 My BP). Thus, the estimated rate of change of DNA from the comparison of *D. picticornis* and *D. heteroneura* or *D. silvestris* is 0.2–0.3%/My. The latter value is closer to estimates made from the comparison of *D. planitibia*, *D. differens*, *D. heteroneura* and *D. silvestris*.

The same difficulty is involved in the estimate of DNA rate of change made by TRIANTAPHYLLIDIS and RICHARDSON (1982) in several species in Hawaiian *Drosophila*. They found the uncorrected lowering of melting temperature between *D. crucigera* tracer DNA and *D. pilimana*, *D. engyochracea*, *D. silvarentis*, *D. picticornis* and *D. mimica* DNA to be 1.7 ± 0.4 , 2.9 ± 0.6 , 3.7 ± 1 , 5.5 ± 0.5 , and $6.6 \pm 7^\circ$ and concluded that the rate of change of DNA in these species was between 1.6 and 5.3%/My. Apart from an obvious error of omission in not estimating total divergence time as twice the time since divergence, it would appear that for many of these species it is impossible to estimate the true time of divergence since there are very few helpful data from morphometric or chromosome inversion studies to construct a meaningful phylogeny. However, from comparisons of several of the species in which phylogeny may be inferred the rate of divergence is likely to be between 0.5 and 1.0%/My. These values are much closer to the ratios presented here, especially since our correction for the change of size of the tracer DNA tends to lower the ΔT_m . In any case it is premature to claim that the rate of change of the DNA of Hawaiian *Drosophila* is any greater than for any other species, as TRIANTAPHYLLIDIS and RICHARDSON (1982) have done.

In a previous study (HUNT, HALL and BRITTEN 1981) we found that the ratio of \bar{D} from protein polymorphism and % DNA difference was nonlinear over 3 or 5 My of divergence. The ratios of \bar{D} and the DNA melting point difference are as follows, using all of the available data: *D. heteroneura*, *D. silvestris*, 0.10; *D. planitibia*, *D. differens*, 0.17; *D. planitibia* or *D. differens* with *D. heteroneura* or *D. silvestris*, 0.21; and for *D. heteroneura* or *D. silvestris* with *D. picticornis*, 0.5.

There is clearly not a linear relationship between the \bar{D} measured for the rather small sample of proteins and the rate of total single copy DNA change as measured by DNA hybridization. Whether this is due to a slower actual rate of change of the DNA in protein-coding sequences in the more recent species or is due to limitations of the neutral mutation hypothesis used to develop \bar{D} is open to question. It would appear, however, that the DNA divergence correlates better with the estimated maximal time of divergence of the species studied here than does the estimates of \bar{D} .

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