Mitochondrial DNA variability in natural populations of Hawaiian *Drosophila*. I. Methods and levels of variability in *D. silvestris* and *D. heteroneura* populations

Rob DeSalle*, L. Val Giddings†,
and Alan R. Templeton

Department of Biology, Washington University,
St. Louis, Missouri, U.S.A.

We describe techniques by which mitochondrial DNA (mtDNA) restriction site information can be obtained for up to 16 different restriction endonucleases on individual Hawaiian *Drosophila*, in particular *D. silvestris* and *D. heteroneura*. We have constructed mtDNA restriction site maps for a total of forty-eight wild caught genomes of both species, from seven major collecting sites on the island of Hawaii. Levels of variability are, in general, high in *D. silvestris* (p of Ewens *et al.*, 1981 = 0.0486) and lower in *D. heteroneura* (p = 0.0327). Measures of population subdivision using Nei's G_{st} indicate that about 50–60 per cent of the observed variability is due to interdemographic subdivision. In accordance, populations within a species show a much lower level of variability, however some populations harbour individuals that are very divergent from the rest of their conspecifics at the same locality. We review two possible mechanisms that could explain the presence of these divergent individuals, hybridisation and the effects of stochastic branching processes.

INTRODUCTION

Analysis of mitochondrial DNA (mtDNA) using restriction endonuclease digestion is useful in the study of evolution in matriarchal lineages (Brown and Wright, 1977; Lansman *et al.*, 1981; Avise *et al.*, 1979; Ferris *et al.*, 1983). The rapid evolution of mtDNA (Brown *et al.*, 1979; Brown, 1980; Avise *et al.*, 1979) means that mtDNA restriction site analysis is also valuable in studying closely related taxa. The Hawaiian *Drosophila* are a well characterised group of closely related organisms in which matriarchal population structure has greatly affected evolution as founder events by one or a few gravid females are probably involved in their extensive genetic differentiation (Carson *et al.*, 1970; Carson and Kaneshiro, 1976; Carson, 1978, 1983; Carson and Templeton, 1984; but also see Barton and Charlesworth, 1984).

The closely related species pair, *D. silvestris* and *D. heteroneura* have undergone extensive morphological and behavioural differentiation as a result of founder events on the island of Hawaii

(Carson, 1983; Kaneshiro, 1983). However, chromosome studies and isozyme analysis show little genetic change within and between these species. *D. silvestris* and *D. heteroneura* are homosequential in their polytene chromosome banding patterns. No diagnostic, fixed inversions exist between the two species nor among populations within each of them (Carson, 1983). Isozyme studies (Craddock and Johnson, 1979; Sene and Carson, 1977) suggest a moderate degree of population heterozygosity, but also show a high degree of genetic similarity not only within populations but between *D. silvestris* and *D. heteroneura*, with no fixed diagnostic alleles between these two generally sympatric species. Hunt and Carson (1982) have used the relatedness of single copy DNA sequences by DNA reassociation techniques to show that small, but significant differences (0.6 per cent sequence divergence) do exist between these two species.

Here we utilise restriction endonuclease cleavage analysis of mtDNA to study genetic differentiation in *D. silvestris* and *D. heteroneura*. We describe techniques that allow detection of restriction site polymorphisms in mtDNA isolated from a single individual Hawaiian *Drosophila* for up to 16 restriction enzymes. In addition, we deter-

Present Addresses: *Department of Genetics, Washington University Medical School, St. Louis, Missouri 63110, †Office of Technology Assessment, United States Congress, Washington, D.C.

mine the level of variability and the degree of genetic differentiation among mtDNA sequences in natural populations of the two species and analyse the patterns of mtDNA variability within populations in light of what is known about population structure (Craddock and Johnson, 1979; Carson, 1983) and natural hybridisation (Kaneshiro and Val, 1977) in these species.

MATERIALS AND METHODS

(i) Flies and fly stocks

Flies were captured by standard Hawaiian *Drosophila* collecting techniques (Carson, 1983) on the western side of Hawaii at Waihaka and Hualalai (fig. 1), and on the eastern side of Hawaii at Olaa and Pihihonau (fig. 1). Males were frozen at -80°C while females were allowed to establish isofemale lines before being frozen. Thirty-two isofemale lines were analysed.

Two males and nine females that did not produce isofemale lines from the west side were analysed, as were four males from the east side (table 1).

Figure 1 Location of the collecting sites of *D. silvestris* and *D. heteroneura* on the island of Hawaii for this study. The solid line running across the map represents the artificial demarcation of the east side from the west side. Contour lines represent elevation in metres. Abbreviations are HU = Hualalai, KA = Kahuku, WA = Waihaka, PI = Pihihonau, KI = Kilauea and OL = Olaa. See table 1 for sample sizes analysed for each population.

Table 1A Isofemale lines used in this study

Isofine	Species	Trapping locality
LVGP1	S	Piihonua—PI
LVGP10	S	Piihonua—PI
LVGP12	S	Piihonua—PI
LVGP13	S	Piihonua—PI
WPMO1	S	Olaa—OL
WPMO4	S	Olaa—OL
WPMO6	S	Olaa—OL
U28T2	S	Kilauea—KI
W12B7	S	Maulua—MA
W33B45	S	Kohala—KO
W48B1	S	Hualalai—HU
W48B2	S	Hualalai—HU
W48G4	S	Hualalai—HU
W48G5	S	Hualalai—HU
W48G10	S	Hualalai—HU
U26B9	S	Kahuku—KA
W33B2	H	Waihaka—WA
W33B3	H	Waihaka—WA
W33B4	H	Waihaka—WA
W33B5	H	Waihaka—WA
W33B7	H	Waihaka—WA
W33B8	H	Waihaka—WA
W33B13	H	Waihaka—WA
W33B14	H	Waihaka—WA
W33B16	H	Waihaka—WA
W33G6	H	Waihaka—WA
W33G7	H	Waihaka—WA
W33G10	H	Waihaka—WA
W33G11	H	Waihaka—WA
W48G3	H	Hualalai—HU
W48B6	H	Hualalai—HU
Q71G12	H	Olaa—OL

S = *D. silvestris*.

H = *D. heteroneura*.

(ii) DNA isolation

D. melanogaster mtDNA used in making probe and in cloning experiments was isolated from embryos by the method of Bultmann and Laird (1973). Nuclear DNA, mitochondrial DNA and RNA were isolated from single Hawaiian *Drosophila* by the method of Coen *et al.* (1981). Plasmid DNA was isolated by methods of Maniatis *et al.* (1982).

(iii) Cloning of a specific Hind III

D. melanogaster mtDNA fragment

Purified mtDNA and puc8 DNA (Vierra and Messing, 1983) were cleaved to completion with Hind III and ligated. The resultant ligated DNA was transformed into *E. coli* JM83 by the method of Maniatis *et al.* (1982). The recombinant plasmid pDm-mt-258 which corresponds to the Hind-C fragment in Bonner *et al.* (1977) was isolated by

Table 1B Single flies analyzed in this study

Designation	Species/sex	Locality
LVGP2	S/F	Piihonua—PI
LVGP8	S/F	Piihonua—PI
LBGP10	S/F(ISO)	Piihonua—PI
LVGP12	S/F(ISO)	Piihonua—PI
LVGP11	S/F	Piihonua—PI
LVGP15	S/F	Piihonua—PI
LVGP16	S/F	Piihonua—PI
LVGP17	S/F	Piihonua—PI
WPMO1	S/F	Olaa—OL
WPMO2	S/F(ISO)	Olaa—OL
WPMO3	S/F	Olaa—OL
WPMO4	S/F(ISO)	Olaa—OL
WPMO5	S/F	Olaa—OL
WPMO6	S/F(ISO)	Olaa—OL
WPMOM1	S/M	Olaa—OL
WPMOM2	S/M	Olaa—OL
W48BA	S/M	Hualalai—HU
W48AA	S/M	Hualalai—HU
W48BC	S/M	Hualalai—HU
W48BD	S/M	Hualalai—HU

S = *D. silvestris*.H = *D. heteroneura*.

F = Female.

F(ISO) = Mother of the designated isoline.

M = Male.

filter colony hybridisation (Maniatis *et al.*, 1982) using purified *D. melanogaster* mtDNA as probe.

(iv) Restriction, electrophoresis, and Southern transfer

Up to 18 different restriction reactions could be performed on DNA from a single fly. Restriction enzymes were purchased from New England Biolabs and used as described by the distributor. Digested DNA was separated on 0.6–1.2 per cent agarose gels and the gels stained with ethidium bromide and photographed under short wave UV light. Restriction fragments were denatured in the gel in 0.5 M NaOH and 1.5 M NaCl for 1 hour and neutralised in 0.5 M Tris and 3 M NaCl for at least 2 hours. Transfer of DNA from the gel to nitrocellulose was done by the Southern blotting technique described in Maniatis *et al.* (1982). Nitrocellulose filters were baked at 65°C for at least 2 hours to affix the DNA to the nitrocellulose before hybridisation.

(v) Nick translation, hybridisation and autoradiography

Purified *D. melanogaster* mtDNA or plasmid DNA was nick translated by the method of Rigby *et al.* (1976) to a specific activity of $5(10^7)$ cpm/ μ g. Approximately $2(10^5)$ cpm of nick translated probe

was hybridised to each filter under the following conditions; 5X SSC, 0.25 M KPB, 2 per cent SDS and 1X Denhardt's at 65°C for at least 12 hours. Filters were washed in 2 changes of 2X SSC, 1 per cent SDS, and 1X Denhardt's at 37°C for 1 hour. Autoradiography was as described in Maniatis *et al.* (1982) for exposure times of 1–10 days.

(vi) Melting point determinations

Melting point determinations were carried out by a modification of the methods in Appels and Dvorak (1982). Three replicates of total cellular DNA (2 μ g) from the appropriate species were denatured in 0.15 ml of 0.2 N NaOH by boiling for 2–5 minutes. The samples were then cooled on ice. Ammonium acetate (0.03 ml of 5 M solution) was then added to the DNA and the solution was dotted onto nitrocellulose. The DNA was baked onto the filter at 80°C for 1–2 hours. Hybridisation of saturating amounts of *D. melanogaster* mtDNA was performed as described above. Determination of the melting point was accomplished by serial transfer of the filters to 0.5 ml aliquots of 2X SSC at the desired temperature for 5–10 minutes at which time the aliquots were placed on ice and the filters transferred to the next vial. To determine the amount of released labelled probe, 5 ml of scintillation cocktail was added and 5 minute counts of each vial were obtained. The activity released was then computed as a cumulative per cent loss of radioactivity.

(vii) Sizing of fragments and restriction mapping

Lambda phage fragments of known size were run on each gel as size standards. Fragments on autoradiographs were sized either by using a computer program designed after the algorithm of Schaffer and Sederoff (1981) or by plotting the lambda standard data on semilog graph paper. The mtDNA restriction maps were generated by single and double digestions of the mtDNA. The restriction enzyme Nru I was particularly useful in the mapping studies. A single invariant site occurs in all of the individuals we examined. This Nru I site is located in the second two codons of the COII gene (DeBruijn, 1983) and was used in all of our double digestion experiments. Orientation of the mtDNA circle was accomplished by using pDm-mt-258 (Hind-C fragment mtDNA) and analogy to known conserved sites in *D. melanogaster* and *D. virilis* (Shah and Langley, 1979) and *D. yakuba* (Clary *et al.*, 1982).

RESULTS

To develop a rapid and efficient system for visualising restriction endonuclease digested mtDNA patterns we used heterologous probes rather than direct visualisation by EtBr staining (Powell, 1983; Powell and Zuniga, 1983). Because Hawaiian picture-winged *Drosophila* are difficult to culture in large numbers, use of tissue to isolate purified mtDNA for probe was not feasible. *D. melanogaster* has mtDNA which hybridises to picture wing mtDNA and is easy to culture in large numbers. In addition, all or part of the mtDNA genome of this species has been cloned so that large scale plasmid or phage isolation of probe is possible (Bonner *et al.*, 1977; di Bruijn, 1983; Clary *et al.*, 1982). We tested the similarity of *D. melanogaster* mtDNA to Hawaiian *Drosophila* mtDNA by making melting point determinations (Appels and Dvorak, 1982) for heterologous and homologous combinations of mtDNA under normal hybridisation conditions (fig. 2). A sigmoidal release of radioactivity with rising temperature indicates that the sequences used behaved as DNA duplexes. Both *D. silvestris* and *D. heteroneura* yielded a delta T_m of about 14°C or approximately 15 per cent nucleotide sequence divergence from *D. melanogaster*. The similarity between the picture-winged mtDNA and *D. melanogaster* mtDNA is hence great enough to allow detection of fragments as small as 500 bp (fig. 4).

Enough mtDNA could be isolated from a single fly for several different restriction endonuclease

digests. Fig. 3 shows the result of digesting nucleic acids isolated by the method of Coen *et al.* (1982) with Xho I, an enzyme which cuts once in the mtDNA circle of *D. silvestris* and *D. heteroneura*. Only one sixth of the DNA isolated from a single fly was run per lane to produce this gel and autoradiograph, and even further dilution of our DNA samples from single individuals produced visible bands of hybridisation. We split the DNA isolated from a single fly into enough aliquots to produce interpretable data for 16 different endonucleases per individual for the flies listed in table 1. Fig. 4 shows an autoradiograph using one eighteenth of the single fly DNA per lane. This approach makes it possible to study populations taken directly from nature without the need for rearing many flies in the lab.

Data for the 34 isofemale lines (table 1) were collected for 23 restriction enzymes, while data on wild caught adults that did not produce isofemale lines (table 1) were collected for 16 endonucleases that were known to cleave the mtDNA circle at least once. Here we report the results of our analysis using these 16 endonucleases.

All restriction sites were mapped by using combinations of single and double digests. They were oriented with *D. melanogaster* mtDNA coding regions by using pDm-mt-258, a cloned probe specific for a region containing the COI, COII, COIII, URF-5 and ATPase 6 genes (Clary *et al.*, 1983). Fig. 5 and table 2 show the restriction endonuclease maps for the mtDNA of *D. silvestris* and *D. heteroneura*. The 16 restriction endonucleases analysed gave 73 mapped restriction sites, of which only 28 were present in all the individuals examined ($n=48$). An average of 44 sites per individual were mapped, with 16 sites per individual being polymorphic. There were 36 distinct mtDNA genomes or haplotypes among the 48 individuals examined.

Levels of variability within a population and a species may be estimated using one of several methods (Brown *et al.*, 1979; Nei and Li, 1979; Ewens *et al.*, 1981; Engels, 1982; Hudson, 1982). The common statistic for the measurement of variability is p , the percentage of sites which are polymorphic in the sample. In order to assess the genetic structure in natural populations of *D. silvestris* and *D. heteroneura* we calculated p values for the *D. silvestris* populations at Olaa ($n=8$), Pihihouna ($n=10$) and Hualalai ($n=9$), and the *D. heteroneura* populations at Waihaka ($n=13$) and Hualalai ($n=2$). Measures of p were also obtained for all *D. silvestris* pooled ($n=32$), all *D. heteroneura* pooled ($n=18$), *D. heteroneura*

Figure 2 Melting curves for homologous and heterologous combinations of mtDNA. (Δ) *D. melanogaster*—*D. melanogaster*; (○) Picture Wing—*D. melanogaster*. The Picture Wing—*D. melanogaster* data points were obtained by combining the data for both *D. silvestris* and *D. heteroneura* because the data for these two species was homogeneous.

Figure 3 Panel A shows DNA prepared by the method of Coen *et al.* (1981), cut with Xho I and electrophoresed on a 0.6 per cent agarose gel. The gel was then stained with ethidium bromide and photographed under short wave UV light. mtDNA can be recovered in relatively large quantities in this type of extraction. Panel B shows an autoradiograph of a Southern blot prepared from the gel in panel A which had been hybridised to nick translated *D. melanogaster* mtDNA. One sixth of the DNA from a single fly was used per lane to produce this gel and autoradiograph. A = U26B9, B = U28T2, C = W12B7, D = LVGP1, E = WPMO1, F = O71G12, S = λ Hind III standard.

from the west side of Hawaii ($n = 17$), *D. silvestris* from the west side ($n = 10$) and *D. silvestris* from the east side ($n = 22$) (table 3).

Takahata and Palumbi (1985) have developed techniques for the estimation of within deme (I) and between deme (J) identity probabilities from restriction maps of mtDNA. These techniques also allow an estimation of relative interdeme gene flow. Table 4 lists the identity probabilities, I and J for pairwise comparisons of the four demes in this study with relatively large sample size. G_{st} values can then be estimated from the within (I) and between (J) identity probabilities in pairwise comparisons. According to Takahata and Palumbi (1985) G_{st} estimates the fraction of genetic variation within a population that is due to interdeme differences. Large G_{st} values for a pairwise comparison suggests a relatively low rate of interdeme gene flow, while small values of G_{st} suggest a high degree of genetic exchange between the demes. The G_{st} value for the four large populations considered together (*D. silvestris* from Olaa, Piionua and Hualalai and *D. heteroneura* from Waihaka) is 0.66, while the G_{st} value for the three *D. silvestris* populations with large sample size is 0.52.

The estimator, p (Ewens *et al.*, 1981) can also be used as a measure of genetic distance among

individuals. Some individuals within these populations were moderately differentiated from the rest of the members of their population. Two divergent *D. silvestris* (WPMO1 from Olaa and W48B2 from Hualalai) are of particular interest. Table 5 shows the genetic distance measures for *D. silvestris* populations at Olaa and Hualalai. W48B2 from Hualalai is two to three times more distant from its conspecifics at Hualalai than is the average *D. silvestris* from this locality. Cluster analysis using the genetic distance measure and UPGMA techniques (Sneath and Sokal, 1973) revealed that W48B2 actually clustered with *D. heteroneura* rather than with *D. silvestris* from Hualalai (Fig. 6(a)). The same is true for *D. silvestris* line U26B9 from Kahuku. Because of the recent divergence of these two species (more recent than the age of the island of Hawaii which is about 500,000 years [McDougall and Swanson, 1972]) convergence of restriction site state is unlikely to explain the high genetic similarity among these individuals (see Templeton, 1983a). Hybridisation between the two species (Ferris *et al.*, 1983; Powell, 1983) and the effects of stochastic branching processes on maternal lineage survivorship (Avise *et al.*, 1983; Avise *et al.*, 1984) as discussed in the next section are two possible explanations for this observation.

Figure 4 Autoradiograph of Hind III cut DNA from several *D. silvestris* individuals. The autoradiograph was produced by hybridising highly purified nick translated *D. melanogaster* mtDNA to a Southern blot of Hind III cut *D. silvestris* DNA. One eighteenth of the total DNA from a single fly was used per lane to produce this autoradiograph. This autoradiograph also demonstrates the ability of this technique to detect very small restriction fragments. A = WPM-M1, B = WPM-M2, C = LVGP2, D = LVGP8 and E = LVGP11.

In contrast to these divergent lines, WPMO1 from Olaa has a genetic distance to its conspecifics that is 2–3 times greater than that observed among its conspecifics from the same locality. Genetic distance between this line and *D. heteroneura* (Q71G12) at Olaa however, is even greater. Cluster analysis reveals no clustering of *D. heteroneura* with *D. silvestris* in the population at Olaa (fig. 6(b)).

DISCUSSION

mtDNA restriction endonuclease data has recently been applied to the population genetics of humans and apes (Brown, 1980; Cann *et al.*, 1982; Ferris *et al.*, 1981), lizards (Brown and Wright, 1979), rodents (Lansman *et al.*, 1981; Brown and Simpson, 1981; Ferris *et al.*, 1983; Avise *et al.*, 1983) and *Drosophila* (Shah and Langley, 1979; Powell, 1983; Powell and Zuniga, 1983). Vertebrate mtDNA is relatively easy to analyse as it is available in relatively large amounts per individual (Giles *et al.*, 1980; Lansman *et al.*, 1981; Ferris *et al.*, 1983). As Powell (1983) points out, however, mtDNA restriction techniques in *Drosophila* systems usually demands that isofemale lines are used to obtain enough tissue to isolate mtDNA. We have established isofemale lines, but have also developed an alternative approach for analysis of Hawaiian *Drosophila* mtDNA, which takes advantage of the large size of the Hawaiian *Drosophila* (10 to 15 times as large as *D. melanogaster*) and extensive similarity between *D. melanogaster* mtDNA and Hawaiian *Drosophila* mtDNA, we can examine restriction patterns of mtDNA from a single fly for a large number of restriction endonucleases.

Figure 5 Restriction site map of *D. silvestris* and *D. heteroneura* mtDNA showing the distribution of conserved and variant sites. The relative map positions for all 23 enzymes that we have used are listed in table 2. The sites generated by BStE II, Bst XI, Nco I, Nru I, Sac II, and Xba I were not included in the analysis of the levels of variation in natural populations. Pst I did not cleave the mtDNA circle of *D. silvestris* and *D. heteroneura*. Gene designations are as follows: U2 = Unidentified reading frame 2, AT RICH = AT base region, S = mt small ribosomal RNA gene, L = mt large ribosomal RNA gene, U1 = Unidentified reading frame 1, CYTO B = cytochrome b, U4 = Unidentified reading frame 4, U5 = Unidentified reading frame 5, U3 = Unidentified reading frame 3, CO III = cytochrome oxidase subunit III, ATP6 = ATPase 6 gene, CO II = cytochrome oxidase subunit II and CO I = cytochrome oxidase subunit I.

Table 2 List of restriction sites and their relative map position from the conserved *Nru*I site in the CO I gene

Enzyme	Site	Map position	Conserved/ polymorphic
<i>Ava</i> II	a1	3.0	<i>p</i>
	a2	4.5	<i>fbl</i>
	a3	4.9	<i>fbl</i>
	a4	6.9	<i>c</i>
	a5	8.6	<i>c</i>
	a6	9.2	<i>p</i>
	a7	11.7	<i>p</i>
	a8	12.7	<i>p</i>
	a10	14.7	<i>p</i>
<i>Bam</i> H I	bl	7.1	<i>fbl</i>
<i>Bcl</i> I	d1	0.3	<i>p</i>
	d2	1.4	<i>p</i>
	d3	1.7	<i>c</i>
	d4	3.5	<i>c</i>
	d5	11.4	<i>c</i>
	d6	12.7	<i>c</i>
	d7	15.7	<i>p</i>
<i>Bst</i> N I	g1	0.2	<i>fbl</i>
	g2	0.5	<i>p</i>
	g3	1.7	<i>p</i>
	g4	5.7	<i>c</i>
	g5	12.4	<i>fbl</i>
	g6	13.1	<i>fbl</i>
	g7	13.9	<i>fbl</i>
	g8	14.6	<i>fbl</i>
	g9	15.4	<i>c</i>
	g10	15.9	<i>c</i>
<i>Cla</i> I	c1	3.5	<i>c</i>
	c2	4.8	<i>c</i>
<i>Eco</i> R I	e1	0.7	<i>p</i>
	e2	4.9	<i>c</i>
	e3	5.9	<i>c</i>
	e4	11.5	<i>c</i>
<i>Eco</i> R V	j1	4.4	<i>fbl</i>
	j2	7.3	<i>p</i>
	j3	11.1	<i>p</i>
	j4	11.9	<i>p</i>
<i>Hind</i> III	h1	2.2	<i>fbl</i>
	h2	2.8	<i>fbl</i>
	h3	3.5	<i>c</i>
	h4	5.6	<i>c</i>
	h5	6.6	<i>p</i>
	h7	10.8	<i>fbl</i>
	h8	11.2	<i>fbl</i>
	h9	11.9	<i>fbl</i>
	h10	13.2	<i>c</i>
	h12	15.9	<i>c</i>
<i>Hpa</i> I	m1	2.4	<i>e</i>
	m2	5.8	<i>p</i>
	m3	7.5	<i>p</i>
	m4	8.1	<i>p</i>
	m5	10.2	<i>p</i>
	m6	12.9	<i>p</i>
<i>Kpn</i> I	k1	8.5	<i>fbl</i>
	k2	16.0	<i>fbl</i>
	k3	16.6	<i>c</i>

Table 2 (*cont.*)

Enzyme	Site	Map position	Conserved/ polymorphic
<i>Pvu</i> II	p1	7.2	<i>p</i>
	p2	8.1	<i>p</i>
	p3	10.7	<i>c</i>
	p4	13.0	<i>c</i>
	p5	15.1	<i>c</i>
	p6	16.1	<i>c</i>
<i>Sst</i> I	s1	4.5	<i>p</i>
	s2	6.7	<i>fbl</i>
	s3	10.6	<i>c</i>
<i>Sma</i> I	t1	8.6	<i>fbl</i>
<i>Stu</i> I	u1	0.5	<i>p</i>
	u2	5.5	<i>c</i>
	u3	15.5	<i>fbl</i>
<i>Xho</i> I	x1	13.2	<i>c</i>
<i>Xmn</i> I	w1	0.0	<i>p</i>
	w2	12.2	<i>c</i>
	w3	13.8	<i>c</i>

Restriction sites that were mapped but not analysed for variability in this study:

<i>Bst</i> E II	f1	1.3	<i>p</i>
	f2	10.1	<i>fbl</i>
<i>Nco</i> I	o1	1.0	<i>p</i>
	o2	4.0	<i>p</i>
	o4	9.5	<i>fbl</i>
	o5	16.5	<i>c</i>
<i>Nru</i> I	n1	0.0	<i>c</i>
<i>Sac</i> II	r1	2.2	<i>fbl</i>
	r2	6.4	<i>c</i>
<i>Xba</i> I	v1	2.7	<i>c</i>
	v2	3.9	<i>p</i>
	v3	6.3	<i>c</i>
	v4	13.2	<i>c</i>
<i>Pst</i> I	no sites		

p = polymorphic; *c* = conserved; *fbl* = polymorphic, but fixed between localities.

We find that, unlike the situation for isozyme and karyotype comparisons, considerable variability in mtDNA exists in most natural populations of these species. In general, the restriction site variants observed in the east side *D. silvestris* populations are more numerous than on the west, occur in only one or a few individuals and are rarely fixed between populations. In the *D. heteroneura* populations and the west side *D. silvestris* populations, the restriction site variants are less numerous, occur in several individuals in a population and are fixed more frequently between populations.

Table 3 The percentage of sites which are polymorphic in the sample (p), the sample size (n) and the variance associated with the estimation of p [$\text{var}(p)$] for several populations with relatively large sample sizes

Species			
Side of Island			
Trapping site	p	n	$\text{var}(p)$
<i>D. silvestris</i>	0.0486*	32	0.00110
West side	0.0306†	10	0.00049
Hualalai	0.0242	9	0.00014
East side	0.0427	22	0.00067
Piihonua	0.0198	10	0.00035
Olaa	0.0246	8	0.00043
<i>D. heteroneura</i>	0.0327	17	0.00053
West	0.0319	16	0.00056
Hualalai	0.0169	2	0.00032
Waihaka	0.0102	13	0.00028

* Significantly different at $p < 0.001$ from the pooled *D. heteroneura* value.

† Significantly different at $p < 0.01$ from the *D. silvestris* east side pooled value.

Table 4 I , J and G_{st} values for four populations of *D. silvestris* and *D. heteroneura* with large sample sizes

	Olaa (S)	Piih (S)	Hual (S)	Waih (H)
Olaa	0.823	0.782	0.681	0.629
Piihonua (Piih)	0.25	0.851	0.669	0.633
Hualalai (Hual)	0.58	0.63	0.906	0.704
Waihaka (Waih)	0.75	0.78	0.81	0.98

Within deme identity or I values for the designated populations are given on the diagonal and are underlined. Between deme identity probabilities or J values are given in the upper part of the matrix. G_{st} values of the pairwise comparisons are given in the lower part of the matrix. I , J and G_{st} were estimated by the procedures outlined in Takahata and Palumbi (1985). Abbreviations: $S = D. silvestris$ and $H = D. heteroneura$.

There is a general similarity between the trends in mtDNA variability and those observed in chromosomal variability of *D. silvestris* and *D. heteroneura*. Table 3 shows that *D. heteroneura* mtDNA is about 30 per cent less variable than *D. silvestris* (p values for *D. heteroneura* and *D. silvestris* are 0.0327 and 0.0486 respectively, which are significantly different at $p < 0.001$ using the Student's t test). The same trend exists for chromosomal data. While *D. heteroneura* has only one polymorphic inversion, *D. silvestris* has 11 (Carson, 1983) and Craddock and Johnson (1979) show that *D. heteroneura* has a maximum of 0.538 heterozygous inversions per individual, while *D. silvestris* has between 0.714 and 2.259 heterozygous inversions per individual.

Figure 6 UPGMA generated phenograms of the Olaa and the Hualalai populations of *D. silvestris*. Abbreviations for Olaa: O1 = WPMO1, O2 = WPMO2, O3 = WPMO3, O4 = WPMO4, O5 = WPMO5, O6 = WPMO6, OM1 = WPM-M1, OM2 = WPM-2 and Q71 = Q71G12. Abbreviations for Hualalai: G3 = W48G3, B6 = W48B6, B2 = W48B2, B1 = W48B1, G4 = W48G4, G5 = W48G5, G10 = W48G10, BA = W48BA, AA = W48AA, BC = W48BC and BD = W48BD.

Furthermore, within *D. silvestris*, the east side populations seem to be more variable than the west side populations for both mtDNA restriction sites and chromosomal inversions. The west side populations have only five polymorphic inversions, while the east side populations have between seven and 11 inversions (Carson, 1983). Similarly, as table 3 shows, the p values for the west side and east side were 0.0306 and 0.0427 respectively, which were significantly different at $p < 0.01$ using the Student's t test. However, isozyme data, show no difference in the levels of variability between the two species (Johnson *et al.*, 1975; Craddock and Johnson, 1979; Sene and Carson, 1977).

The G_{st} values (table 4) for the comparisons of the four relatively large samples in this study allow an estimate of the levels of gene flow. The relatively large G_{st} value for all the populations considered together ($G_{st} = 0.66$) indicates that about two-thirds of the observed variation in mtDNA can be attributed to interdeme variation. Similarly, the relatively large G_{st} value ($G_{st} = 0.55$) indicates a large amount of subdivision within *D. silvestris*. This pattern of population subdivision is also observed in all the pairwise G_{st} comparisons,

Table 5A *P* estimates for comparisons within the Olaa *D. silvestris* populations. All individuals are listed in tables 1 and 2 and are listed in this table without the WPM prefix. A single *D. heteroneura* from the same locality, Q71G12 (abbreviated as Q71 in this table) is also listed

	O1	O2	O3	O4	O5	O6	OM1	OM2	O71
O1	—	0.019	0.013	0.015	0.019	0.017	0.017	0.019	0.026
O2		—	0.013	0.008	0.004	0.006	0.010	0.012	0.026
O3			—	0.013	0.013	0.011	0.008	0.006	0.018
O4				—	0.008	0.009	0.010	0.008	0.026
O5					—	0.009	0.010	0.008	0.026
O6						—	0.008	0.013	0.024
OM1							—	0.010	0.021
OM2								—	0.020
Q71									—

Table 5B *P* estimates for comparisons within the *D. silvestris* Hualalai population. All individuals are listed in tables 1 and 2 and are listed in this table without the W48 prefix. Two *D. heteroneura* from the same locality are also listed in this table (W48G3 abbreviated as G3 and W48B6 abbreviated as B6)

	B1	B2	G4	G5	G10	BA	AA	BC	BD	G3	B6
B1	—	0.020	0.007	0.005	0.007	0.005	0.010	0.007	0.005	0.025	0.029
B2		—	0.017	0.016	0.014	0.016	0.011	0.017	0.016	0.019	0.011
G4			—	0.002	0.003	0.002	0.007	0.000	0.002	0.019	0.024
G5				—	0.002	0.000	0.005	0.002	0.000	0.020	0.025
G10					—	0.002	0.007	0.004	0.002	0.019	0.023
BA						—	0.005	0.002	0.000	0.020	0.025
AA							—	0.007	0.005	0.022	0.021
BC								—	0.002	0.019	0.024
BD									—	0.020	0.025
G3										—	0.017
B6											—

except for the Olaa and Pihihouna *D. silvestris* comparison. The low G_{st} value for this comparison may reflect a high degree of interdeme contact between these populations. Whether this results from migration or is caused by recent divergence of these two populations cannot be determined by this analysis. The G_{st} values given in table 5 also indicate that the degree of isolation of the east side *D. silvestris* (Olaa and Pihihouna) from the west side *D. silvestris* (Hualalai) is nearly the same as the degree of isolation from *D. heteroneura* (Waihaka). This suggests that there is extensive differentiation of the east and west side *D. silvestris*.

In contrast to the chromosomal and isozyme data (where there are no fixed variants between populations and between species) there are several restriction site variants which are fixed between the two species and even among populations within a species. The fixed differences within *D. silvestris* are found only between the east side populations and the west side populations.

The presence of genetically distinct individuals within the *D. silvestris* populations (table 5 and

fig. 6) suggests a relatively complex population structure for this species. First, *D. silvestris* individuals are capable of hybridising in nature with *D. heteroneura*. Kaneshiro and Val (1977) have presented morphological data on natural hybridisation and mtDNA provides genetic data supporting the possibility of hybridisation. Chromosomal (Craddock, 1974) and isozyme data (Sene and Carson, 1977; Craddock and Johnson, 1979) on the west side *D. silvestris* and *D. heteroneura* populations, however indicate that the two species at single localities are relatively dissimilar, and the isozyme data supports the contention that hybridisation is limited at least in the populations studied by Craddock and Johnson (1979).

If hybridisation has occurred between *D. silvestris* and *D. heteroneura*, its impact on the genetic structure of the species involved is not the same as in other animals where hybridisation has been inferred from mtDNA analysis (Ferris *et al.*, 1983; Powell, 1982) and in which there are distinct differences between the nuclear genomes of the

species involved. In these cases mtDNA gene pools of hybridizing species become homogenized, with loss of one of the mtDNA lineages. In the *D. silvestris* and *D. heteroneura* system little differentiation has occurred in the nuclear genome (Johnson *et al.*, 1975; Sene and Carson, 1978; Craddock and Johnson, 1979; Carson, 1983; Hunt and Carson, 1982), but the mtDNA pools of *D. silvestris* and *D. heteroneura* have not been homogenised.

The genetic similarity observed between some west side *D. silvestris* and *D. heteroneura* individuals may alternatively be related to the stochastic branching processes involved in matriarchal lineage survivorship (Avise *et al.*, 1983; Avise *et al.*, 1984). If the mtDNA lineages giving rise to W48B2 (*D. silvestris* from Hualalai) and U26B9 (*D. silvestris* from Kahuku) came from the *D. silvestris*/*D. heteroneura* split, then these divergent *D. silvestris* lineages would appear more closely related to *D. heteroneura* than to the other *D. silvestris* lineages. Avise *et al.* (1984) have shown that for very young populations or species some of the mtDNA lineages within the population or species will have originated before separation from the ancestral lineage. Since the populations and species in this study are certainly young, this is a plausible explanation.

The presence of divergent individuals in the *D. silvestris* populations that are not suspected of being hybrids with *D. heteroneura* might result from such processes; but might also result from migration, strong local population subdivision or both. More detailed collecting and mtDNA restriction site analysis are needed to resolve this question.

The present data on mtDNA restriction sites show a high degree of variability within and between populations. Many restriction sites are fixed between populations and between the two species, allowing a genetic analysis of their relationships to be made. Such an analysis using distance methods (Ewens *et al.*, 1981) and non-parametric phylogeny testing methods (Templeton, 1983a, b) appears in the accompanying paper.

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