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Intraspecific and interspecific variation in the sequence and abundance of highly repeated DNA among mosquitoes of the *Aedes albopictus* subgroup

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Interspecific variation in abundance of highly repeated DNA sequences has been examined in three species of the *Aedes scutellaris* and three species of the *A. albopictus* subgroup of the *A. scutellaris* group. Sequences from a population of *A. albopictus* were hybridised with whole genomic content from other species and strains. Copy number estimates were determined by dot-blot hybridisation. Variation in sequence abundance between strains of *A. albopictus* was as great as between it and the other six species. Two clusters were formed by principal components analysis, each of which contains populations of *A. albopictus*. Copy numbers of highly repeated sequences do not correlate with genome size. The results indicate extensive sequence divergence and rapid evolution. These findings are discussed in relation to the importance of concerted evolution and natural selection in the evolution of the species group.

INTRODUCTION

Most eukryote genomes contain hundreds or thousands of families of highly repeated DNA sequences (John and Miklos, 1979; Brutlag, 1980; Bouchard, 1982) varying in sequence length from ten to several thousand base pairs (Maresca *et al.*, 1984; Skowronski *et al.*, 1984) and constituting up to 80 per cent of the genome (MacGregor, 1982). Variation in both sequence (Pages and Roizes, 1984; Maresca *et al.*, 1984) and abundance (Dover, 1981; Singer, 1982; Bouchard, 1982) of highly repeated sequences occurs between closely related species. Some sibling species of *Drosophila* differ by an order of magnitude in the abundance of dispersed repetitive DNA (Dowsett and Young, 1982; Strachan *et al.*, 1982). Changes in abundance may have important evolutionary consequences through indirect effects on developmental time (Cavalier-Smith, 1982) and rates or localisation of recombination (John and King, 1985).

The present study examines interspecific variation in the abundance of highly repeated DNA sequences in three species of the *Aedes scutellaris* subgroup and three species of the *A. albopictus* subgroup of the *A. scutellaris* group. The *A. scutellaris* group consists of 34 species in the *scutellaris* subgroup, confined primarily to Polynesia and Melanesia, and 11 African, Asian, and Pacific species in the *albopictus* subgroup (Rai *et al.*, 1982). Also, intraspecific variation in sequence abundance, which has received little study (Maresca *et al.*, 1984), is examined in 15 strains of *A. albopictus* derived from geographic locations extending from Madagascar through India, southeastern Asia, and the Pacific. Within the *A. scutellaris* group genome size varies from less than 0·5 pg to over 1·3 pg per haploid genome (Rao and Rai, 1986), with moderately-highly repeated sequences accounting for the variation (Black and Rai, in preparation).

Species of the two subgroups diverged approximately 10 million years ago as judged from allozyme variation and geologic history (Pashley and Rai, 1983). In the *A. scutellaris* subgroup, *A. malayensis* appears to have diverged from *A. hebrideus* 2–3 million years ago, and from *A. pseudoscutellaris* 7–10 million years ago (Pashley *et al.*, 1985). *A. hebrideus* and *A. pseudoscutellaris* appear to have diverged about 5 million years ago (Pashley *et al.*, 1985). Phylogenetic relationships within the *A. albopictus* subgroup have yet to be elucidated, although *A. pseudalbopictus* and *A. seatoi* are probably more closely related to each other than to *A. albopictus* while *A. flavopictus* is probably relatively distantly related to the other three species (Rai *et al.*, 1982). Species within the

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A. albopictus subgroup reflect a recent radiation relative to the *A. scutellaris* subgroup (McLain and Rai, 1986; McLain *et al.*, 1985).

The objectives of the present study are to determine if changes in sequence and abundance: (a) evolve rapidly, (b) promote postmating reproductive isolation, and (c) are responses to selection pressure. The experimental approach was to hybridise highly repetitive sequences isolated from a single population of *A. albopictus* (Oahu, Hawaii, U.S.A.) to whole genomic DNA from other species and strains. Copy number estimates of the sequences were determined through quantitative dot-blot hybridisation. Sequence variation was revealed by differential hybridisation at low versus high stringency.

METHODS

DNA isolation and cloning

DNA was isolated from larvae or pupae by the procedure of Fleming *et al.* (1983), as modified from Fuchs and Green (1979). Following ethanol precipitation DNA was resuspended in sterile water and stored at 4°C.

DNA from *A. albopictus* (Oahu strain) was digested for 2 hours with restriction enzyme Eco RI (Bethesda Research Laboratories [BRL], Gaithersburg, MD, U.S.A.; used 10 units/μg DNA) and ligated to restricted plasmid pUC 12 which contains an Eco RI site within a lac Z gene (Vieira and Messing, 1982) permitting selection of recombinants on LB media coated with 2 per cent bluo-gal (from BRL) in dimethyl formamide. Ligation and subsequent transformation into *E. coli* strain JM83 was performed as described in Maniatis *et al.* (1982). Transformants were selected on media containing 50 μg ampicillin/ml.

Detection of repetitive sequences

Clones of length 1000–7000 base pairs were nick translated (BRL kit #81065B) to incorporate labelled deoxycytidine and then hybridised to Hind III digested (10 units/μg DNA for 12 hours; enzyme from BRL), whole genomic DNA immobilised on nitrocellulose paper (Southern, 1975). Transfer of DNA from a 1 per cent agarose gel to nitrocellulose was as described in Smith and Summers (1980). Hybridisation was performed under stringent conditions (50 per cent formamide, 2×SSC) with washing at 65°C (Smith and Summers, 1980). Autoradiography utilised Kodak XRP-1 film intensified with a Dupont Cronex Lightening Plus screen at −80°C. Repeated sequences yielded a smear of hybridisation (fig. 1) while low copy sequences produced a single band or no detectable hybridization. Eight clones ranging in size from 1000–7000 base pairs containing highly repeated sequences were thus identified. None of these clones cross-hybridizes at low stringency (10 per cent formamide, 5×SSC, washing at 42°C). Restriction mapping of clones has revealed no tandem repetition of sequences (Ferrari and Rai, in preparation), indicating, perhaps, that the repetitive sequences in the clones are dispersed.

Variation in abundance and dot-blot hybridization

Fifteen μg of unrestricted, RNase A-treated, genomic DNA in 2 M sodium acetate was dotted (Kafatos *et al.*, 1979) onto untreated nitrocellulose paper using a dot-blot minifold (Schleicher and Schuell, Keene, NH, U.S.A.). For each nick-translated probe, DNA from all 21 strains and species was simultaneously hybridised on the same sheet of nitrocellulose. Differences in the abundance of a repeated sequence were revealed by differences in the intensity of autoradiographic dots (fig. 1), and quantified through densitometry tracings which yield a series of peaks each corresponding in size to the intensity of a particular dot. There is a linear relationship between the intensity of a dot and the amount of mosquito DNA bound on nitrocellulose (McLain *et al.*, 1986; see also Small *et al.*, 1982). Dot hybridisation has proven effective in detecting differences in sequence abundance in numerous other studies (*e.g.*, Collins and Groudine, 1982; Hamada and Kakunaga, 1982; Law *et al.*, 1982).

The copy number estimates for sequences in the genome of *A. albopictus* (Oahu strain) were calculated via the procedure of Brandsma and Miller (1980; see also Hamada and Kakunaga, 1982) by dotting 300 ng of plasmid DNA ($=3\times10^{12}$ copies) alongside 15 μg of mosquito genomic DNA ($=1{\cdot}5\times10^{7}$ copies of the genome; note, 1 pg of DNA $=10^{9}$ base pairs [Strauss, 1971]). Dot intensities were then compared from hybridisations with each cloned sequence (plasmid vector + repeating sequence). Copy numbers could be estimated for other species and strains by direct comparison with Oahu of dot intensity and genome size (Rao and Rai, 1986).

Isolation of DNA for dot blotting, dot blot hybridisations, and copy number estimates were determined twice. The copy number estimates were very consistent (relative to variation between

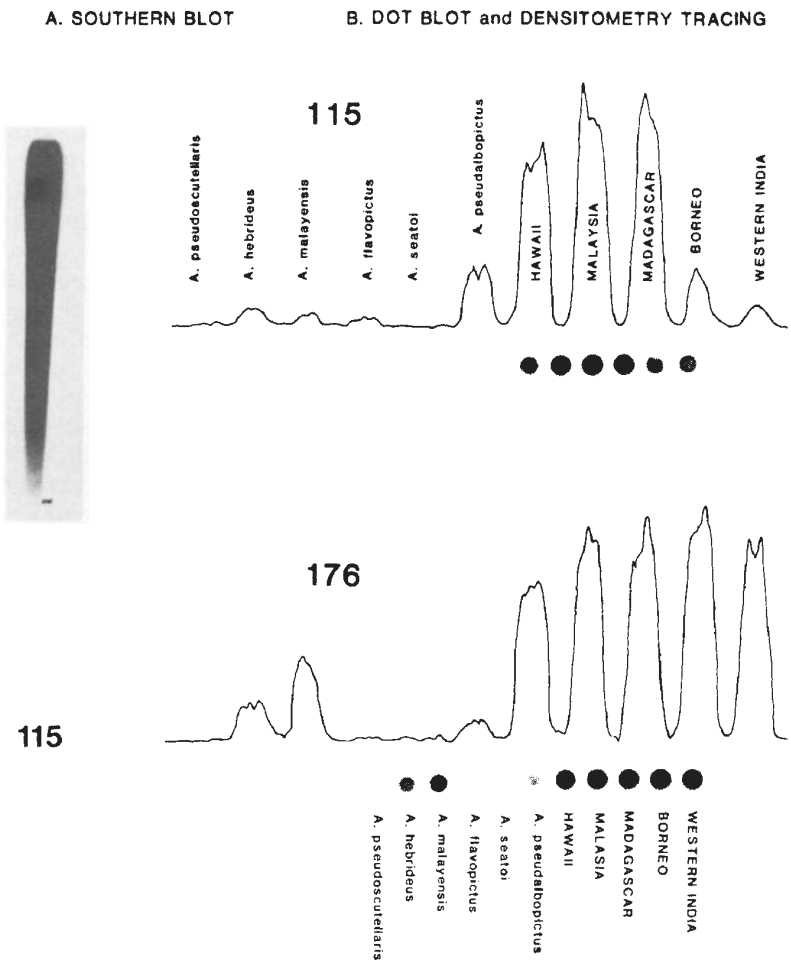

Figure 1 A. Autoradiograph of total genomic DNA digested with Hind III, Southern blotted to nitrocellulose, and then hybridised with a clone (H-115) containing highly repeated sequences. B. Dot blots and densitometry tracings of those dots representing the variation in abundance in seven species (including five strains of *A. albopictus*) of two clones of highly repeated sequences (H-115 and H-176).

populations), varying by 10–20 per cent for clones with copy numbers of at least 10,000 and by 20–30 per cent for clones with copy numbers of less than 10,000. Therefore, the data are presented as averages.

Some error in estimates of sequence abundance may occur if sequence variation affects the efficiency of hybridisation for those species hybridising to Oahu probes at high stringency. Also, it is not known how much sequence variation exists in these species. Since highly repeated sequences evolve rapidly through single base substitution (Pages and Roizes, 1984), a given family may be represented by different sequence variants (*e.g.*, Dover, 1982; Dover *et al.*, 1982). Thus, sequence variation could partially obscure differences in abundance. This variation is potentially important since the *A. albopictus* and *A. scutellaris* subgroups diverged approximately 10 million years ago (Pashley *et al.*, 1985; Pashley and Rai, 1983). However, it is unlikely that there is much sequence variation within a family within a given population since the rate of homogenisation of a variant is high relative to the mutation rate (Strachan *et al.*, 1985).

To test for the applicability of dot-blotting to estimate copy number, species and strains hybridising to Oahu probes at high stringency were also hybridised at low stringency. The abundance of sequences relative to the Oahu genome was compared as a function of stringency. With the exception of *A. albopictus* strains from central

India, Thailand, and the Solomon Islands, the abundance of sequences in all the other strains did not vary by more than 20–30 per cent at high and low stringency. This is within the error for different replicates at high stringency. Since copy number estimates did not vary as a function of stringency, we conclude that sequence variation does not significantly affect estimates of copy number.

Species utilised.

DNA was cloned from *A. albopictus* from Oahu for several reasons. First, the population is at the eastern edge of the geographic range of the species. Therefore, other strains used vary greatly in geographic proximity. Second, several other strains are available from Hawaii permitting the analysis of differences between populations that are, presumably, closely related. Third, the Oahu strain has a relatively large genome size (Rao and Rai, 1986), suggesting an abundance of repeated sequences.

Table 1 lists the date of colonisation of all strains and species. Fig. 2 depicits the species' geographic distributions.

Table 1 Dates of colonisation and sources of species and strains

Species (*albopictus* subgroup)	Date	Source
Aedes albopictus	1966	Saigon, Viet Nam
	1968	Surot Thani, Thailand
	1971	Oahu, Hawaii
	1973	Kuala Lumpur, Malaysia
	1974	Tananareve, Madagascar
	1977	Taipei, Taiwan
	1977	Tokyo, Japan
	1978	Kalimantan, Indonesia (Borneo)
	1978	Hong Kong
	1979	Korea
	1981	Kahoolawe, Hawaii
	1982	Guadal Canal, Solomon Islands
	1984	Pune, India (Central India)
	1984	Coonor, India (West. India)
	1984	Kauai, Hawaii
A. pseudalbopictus	1981	Chiayi Hsien, Taiwan
A. seatoi	1972	Bangkok, Thailand
A. flavopictus (*scutellaris* subgroup)	1981	Nagasaki, Japan
A. pseudoscutellaris	1979	Suva, Fiji
A. hebrideus	1982	South Pentecost
A. malayensis	1968	Prachaup, Thailand

RESULTS

Estimated copy numbers for the eight cloned highly repeated sequences within the genome of the Oahu strain of *A. albopictus* range from 3900 (sequence H-176) to 470,000 (sequence H-85) (table 2). Between populations of *A. albopictus* copy numbers varied significantly for any given sequence (table 2). For instance, sequence H-85 was not detected in the population from Japan (high stringency hybridisation) but contains an estimated 2·6 million copies in the genome of the population from Thailand.

The abundance of the eight highly repeated sequences in *A. albopictus* (Oahu strain) was significantly ($P < 0{\cdot}05$), positively correlated with the abundance in three other *albopictus* subgroup species (*A. pseudalbopictus*, $r = 0{\cdot}912$; *A. seatoi*, $r = 0{\cdot}997$; *A. flavopictus*, $r = 0{\cdot}995$ if sequence H-85 is omitted, otherwise, $r = 0{\cdot}432$, $P > 0{\cdot}05$). Correlations between abundance in *A. albopictus* (Oahu) and the three *scutellaris* subgroup species are significant ($P < 0{\cdot}05$) only when sequence H-85 is omitted (*A. malayensis*, $r = 0{\cdot}417$ or $r = 0{\cdot}995$ omitting H-85; *A. hebrideus*, $r = 0{\cdot}418$ or $r = 0{\cdot}996$ omitting H-85; *A. pseudoscutellaris*, $r = 0{\cdot}413$ or $r = 0{\cdot}928$ omitting H-85).

Three of the repeated sequences (in clones H-19, H-85, and H-115) did not hybridise to some strains of *A. albopictus* or other species under stringent conditions (table 2). However, in every instance hybridisation occurred at low stringency.

The variation in sequence abundance between strains of *A. albopictus* was as great as that between *A. albopictus* and the six other species. In a principal components analysis (fig. 3), two clusters were formed from the plot of the first two principal components, accounting for 84·3 per cent of the variation in the data. Each cluster contains populations of *A. albopictus* and other species from both subgroups. Only a few populations of *A. albopictus* are not present in one or the other cluster.

The seven strains and species in the lower cluster contain relatively low copy numbers of the repeated sequences. The mean of the sum of the copy number for all eight sequences for these seven strains and species is 94,000 ($SD = 56{,}000$). The mean of the eight strains and species of the upper cluster (mean = 545,000, $SD = 144{,}000$) is significantly greater ($P < 0{\cdot}05$; $t = 8{\cdot}16$) than the mean for the lower cluster. The lower cluster consists entirely of island populations. Six of the eight populations in the upper cluster are from Malaysia or mainland and large islands immediately adjacent.

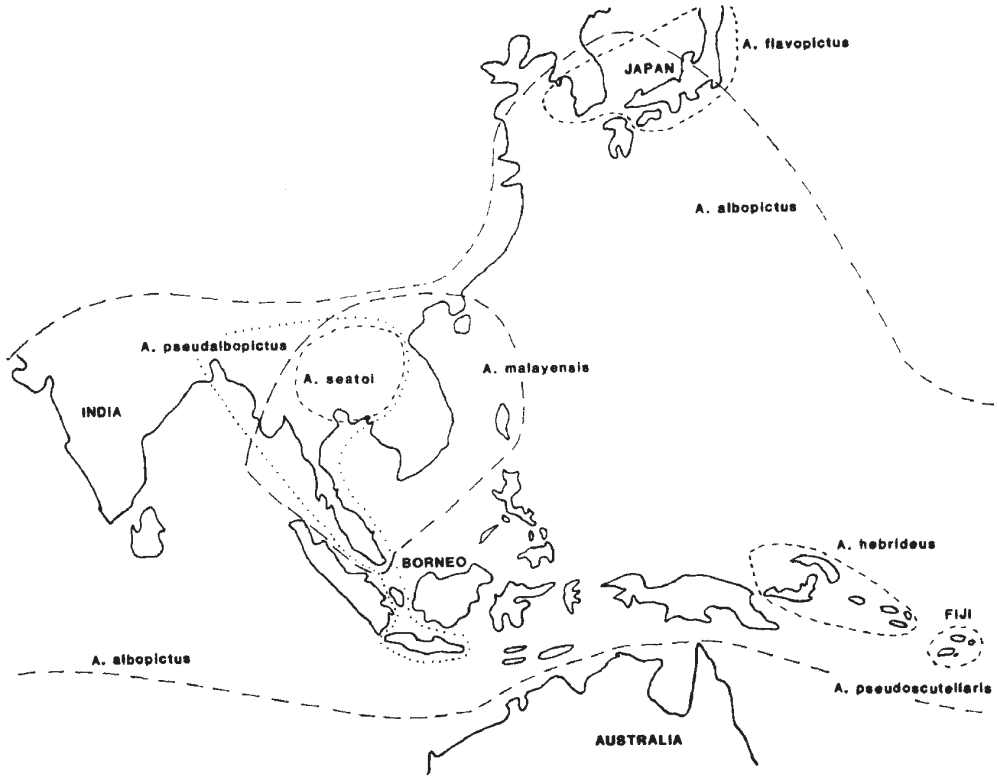

Figure 2 Geographic distribution in the Pacific and south-eastern Asia of the seven *A. scutellaris* group species employed in the present study.

Table 2 Abundance of eight highly repeated sequences in fifteen strains of *A. albopictus* and six other species in the *A. scutellaris* group

Species/strain	Sequence H-19	H-41	H-61	H-75	H-76	H-85	H-115	H-176
A. albopictus								
Oahu	11,000	310,000	19,000	19,000	140,000	470,000	5,800	3,900
Kahoolawe	0	2,600	1,800	1,100	4,000	72,000	0	2,200
Kauai	0	5,900	2,200	1,100	11,000	130,000	3,000	2,700
Solomon Islands	4,900	160,000	15,000	17,000	140,000	160,000	3,200	14,000
Japan	590	4,200	1,200	2,400	3,900	0	8,700	16,000
Hong Kong	920	2,000	920	100	6,400	110,000	21,000	15,000
Taiwan	240	510	1,800	190	17,000	69,000	9,900	18,000
Korea	1,300	160,000	4,600	11,000	160,000	150,000	16,000	17,000
Viet Nam	1,800	61,000	6,200	6,400	59,000	810,000	32,000	29,000
Thailand	5,500	150,000	15,000	13,000	100,000	260,000	39,000	81,000
Central India	4,300	280,000	9,600	3,500	90,000	55,000	480	4,400
Western India	5,600	18,000	3,600	4,500	17,000	93,000	7,500	27,000
Malaysia	2,800	320,000	18,000	8,100	120,000	250,000	3,400	6,000
Borneo	6,200	200,000	7,400	5,800	100,000	290,000	1,100	9,100
Madagascar	7,100	280,000	10,000	8,200	110,000	270,000	6,800	8,800
A. pseudalbopictus	750	280,000	16,000	11,000	110,000	210,000	2,000	560
A. seatoi	3,500	150,000	11,000	7,300	86,000	240,000	0	0
A. flavopictus	0	58,000	1,400	100	20,000	0	180	0
A. malayensis	2,500	250,000	17,000	12,000	87,000	0	120	2,700
A. hebrideus	2,200	190,000	12,000	8,900	68,000	0	430	410
A. pseudoscutellaris	260	19,000	1,700	400	1,400	0	0	0

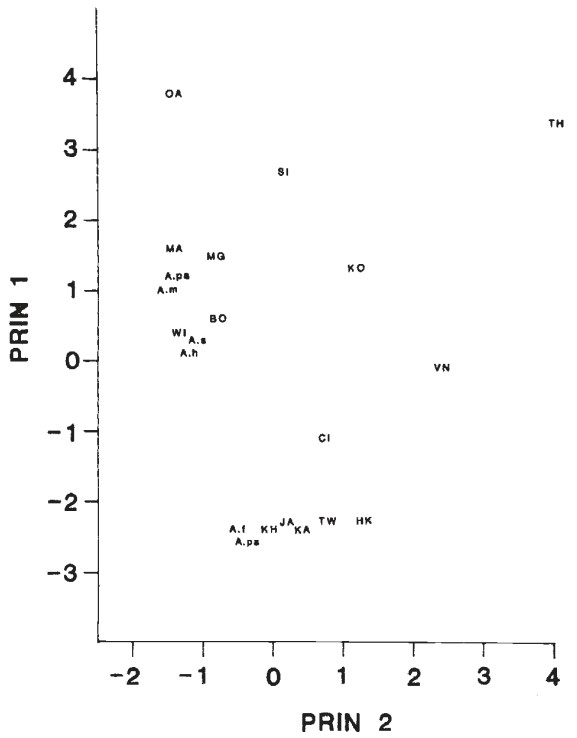

Figure 3 Clustering of the fifteen strains of *A. albopictus* and six other species produced from the plot of the first two principal components (explaining 84·3 per cent of the variation) resulting from a PCA on the abundances of eight highly repeated sequences. Lower cluster: A. f = *A. flavopictus*; A. ps = *A. pseudoscutellaris*; and *A. albopictus* strains, KH = Kahoolawe (Hawaii), JA = Japan, KA = Kauai (Hawaii), TW = Taiwan, and HK = Hong Kong. Upper cluster: A. hs = *A. hebrideus*; A. s = *A. seatoi*; A. m = *A. malayensis*, A. pa = *A. pseudalbopictus*; and *A. albopictus* strains, WI = western India, BO = Borneo, MG = Madagascar, and MA = Malaysia. Other non-clustered *A. albopictus* strains, VT = Viet Nam, CI = central India, TH = Thailand, KO = Korea, SI = Solomon Islands, and OA = Oahu (Hawaii).

None of the highly repeated sequences have copy numbers which correlate significantly with genome size (data from Rao and Rai, 1986) in regression analyses employing: (a) fifteen strains of *A. albopictus*, (b) all 21 strains and species, or (c) the three *scutellaris* and three *albopictus* subgroup species (excluding *A. albopictus*) (table 3).

Table 3 Correlation (Pearson's linear) between the abundance of a highly repeated sequence and genome size. For each sequence the correlation is determined for strains of *A. albopictus* ($n = 15$), all strains of *A. albopictus* plus the six other *scutellaris* group species ($n = 21$), and the *scutellaris* group species excluding *A. albopictus* ($n = 6$)

Sequence	*A. albopictus* strains	Strains and other species	Species excluding *A. albopictus*
H-19	0·024	0·174	−0·178
H-43	−0·347	−0·169	0·311
H-61	−0·491	−0·272	0·276
H-75	−0·039	0·017	0·257
H-76	0·308	0·286	0·466
H-85	−0·234	−0·002	0·348
H-115	−0·370	0·048	0·644
H-176	−0·253	−0·163	−0·013

DISCUSSION

The highly repetitive component of the genome evolves very rapidly. This is supported by: (a) extensive variation in abundance between sibling species in both the *A. scutellaris* and *A. albopictus* subgroups, and (b) variation within the single species, *A. albopictus*, which is as great as that between species. Evolution within the highly repetitive component of the genome has occurred through sequence amplification or diminution and through sequence variation. Extensive sequence divergence is revealed by hybridisation at low stringency following failure to hybridise at high stringency (which requires more precise base pairing). Other studies used this approach to document sequence divergence between sibling species (Strachan *et al.*, 1982; Jeffreys, 1982).

The occurrence of sequence variation between sibling species or between populations of a single species indicates that some cytological processes such as unequal crossing over or gene (sequence) conversion are homogenising populations as different mutants arise (Dover, 1982; Nagylaki and Petes, 1982; Nagylaki, 1984). Such concerted evolution (Dover, 1982; Dover *et al.*, 1982; Dover, 1986) can result from a bias toward a sequence variant in gene (sequence) conversion, promoting the rapid fixation within a population of the preferred variant (Ohta and Dover, 1983 and 1984; Ohta, 1985). Rapid concerted evolution is indicated in the present study by tremendous intraspecific variation in the abundance of different sequence variants. Variation within a population is probably of limited extent since the process of replacing one sequence variant with another (molecular drive) by gene conversion, unequal crossing-over, or through stochastic gain-and-loss conversion is a slow process relative to the rate of random distribution of chromosomes which accompanies sexual reproduction (Ohta and Dover, 1984). An interesting consequence of this is that selection differentials between individuals will be too small for

natural selection to oppose the spread of a variant even if it reduces mean population fitness.

The PCA clustering may indicate selection pressure, perhaps imposed by habitat, for the maintenance of particular abundance profiles of the highly repeated sequences. Thus, there is clustering of geographically contiguous but taxonomically diverse species. For instance, six of eight species and populations in one cluster are from Malaysia or adjacent areas. These include two strains of *A. albopictus* (from Borneo and Malaysia), two other *albopictus* subgroup species, *A. pseudalbopictus* and *A. seatoi* (from Malaysia and Thailand, respectively), and two *scutellaris* subgroup species, *A. malayensis* and *A. hebrideus* (from Malaysia and Melanesia, respectively). The abundance profiles of species in this cluster appear stable since species in the two subgroups have probably been isolated for 10–15 million years (Pashley *et al.*, 1985). It is unlikely that there has been horizontal transfer (introgression) of sequences since there is complete post-mating and complete or significant pre-mating isolation between species (McLain *et al.*, 1985; McLain and Rai, 1986). Similarly, for six species of the *A. scutellaris* subgroup the abundance profiles of nine highly repeated sequences (cloned from *A. malayensis*) are very similar among species pairs occurring on small islands in Polynesia, large islands in Melanesia, or continents (Asia and Australia). Again, these similarities were not associated with well established phylogenetic relationships among the species (McLain *et al.*, 1986).

These associations suggest the action of natural selection. However, the interaction between selection and forces responsible for changes in sequence or copy number of repetitive DNA is complex (Dover, 1986). This complexity arises in part because natural selection is blind to the small differentials between individuals (Dover and Ohta, 1984), as previously outlined. Therefore, the genome need not reflect adaptation to the environment. Instead, it may reflect "adoptation" (Dover, 1986). "Adoptation" occurs if internally driven changes in the genome are not counterpoised by molecular coevolution, destroying adaptation to a given environment (which usually results in extinction) but permitting the population to adjust its niche and utilise a new environment.

Thus, in the present example of the *A. scutellaris* group, the association between habitat and abundance profiles may have resulted from the extinction of all populations with variant abundance profiles of the repetitive sequences. Therefore, the geographic distribution of these species appears to reflect a response, adaptation or adoptation, to genome evolution.

The selective importance of highly repeated sequences can be inferred from: (a) clinal variation in abundance (reviewed in John and Miklos, 1979), (b) correlation between the presence and amount of variable blocks of highly repetitive satellite DNA and the localisation and frequency of chiasmata (John and King, 1985), the higher frequencies being associated with less phenotypic variance (Miklos, 1982), (c) correlation between karyotype diversity and the diversity of highly repeated sequences (Gillespie *et al.*, 1982) and (d) indirect regulation of developmental rates through (i) regulation of transport across the nuclear membrane (Cavalier-Smith, 1978 and 1982; but see Miklos, 1982) or (ii) hierarchical control of gene regulation (Bennet, 1982).

In the present study, PCA also clustered three species (representing both subgroups), including five populations of *A. albopictus*, all of which are restricted to islands dispersed throughout the Pacific. Here, the abundance of most sequences was relatively low. This may indicate that the colonisation of islands was followed by independent, rapid evolution of highly repeated sequences. Rapid genome evolution is also indicated by: (a) the 30 per cent greater genome size of island versus continental populations of *A. albopictus* and (b) the positive correlation between genome size and the amount of sequence variation within highly repeated DNA families (Flavell, 1982). Since the amount of variation within *A. albopictus* is large in comparison to that within the whole group, the genome of this species apparently evolves relatively rapidly. This in turn suggests a sequence-dependent bias in the conversion events which homogenize populations for sequence variants of a repetitive DNA family.

Despite any selective significance inferred from PCA clustering, the highly repeated sequences do not appear to have any direct role in speciation. This is indicated by intraspecific variation which at least equals interspecific variation and which is not associated with postmating reproductive isolation between *A. albopictus* populations (McLain and Rai, 1986). Other studies have also failed to implicate the abundance of highly repeated sequences in reproductive isolation (Miklos, 1982; John and Miklos, 1979). However, this does not preclude an indirect role in either speciation or local adaptation, as discussed above. Of course, we cannot rule out that some key families may have greater importance. Also, sequence composition may be relatively more important than copy

number, especially since concerted evolution (homogenisation of different variants) appears to be a between-species phenomenon (Dover, 1986).

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