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Mitochondrial DNA variability in *Drosophila simulans*: quasi absence of polymorphism within each of the three cytoplasmic races

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Nucleotide variability of mtDNA extracted from 144 isofemale lines collected in the whole range of *D. simulans* was analysed with 10–15 restriction enzymes and 73 lines were studied using one or a few enzymes. All clones were distributed into 3 mitochondrial genomes, *si*I, *si*II and *si*III. These types are allopatric and can define geographic races. Mixed populations occur only in Madagascar and Réunion, where *si*II and *si*III are found together. Among 40 sites detected with 10 enzymes, the variability of the coding region is extremely low, with one or no polymorphic restriction sites depending on the type. The control A+T-rich region is more variable in length and in restriction sites, and allows subtypes to be designated. Several lines were heteroplasmic for the length of the genome. These results are relevant to the evolutionary history of the species, its recent worldwide extension and to probable founder effects at the origin of each of the three types.

INTRODUCTION

Drosophila melanogaster and *D. simulans* are cosmopolitan, commensal and generalist colonizing species. They probably originated from a common Afrotropical ancestor a few million years ago (Lachaise *et al.*, 1988) and have spread to most of the world in the recent past with the aid of human transport (David and Tsacas, 1981; Parsons, 1983). *D. melanogaster* shows great geographic variation (Lemeunier *et al.*, 1986) and such "racial" differentiation may have allowed the colonization of places with very different climates. *D. simulans* is less studied but, for several traits, such as chromosomal polymorphism (Ashburner and Lemeunier, 1976), allozyme frequencies (Hyytia *et al.*, 1985; Singh *et al.*, 1987) thoracic trident pigmentation (Capy *et al.*, 1988) and alcohol tolerance (David and Bocquet, 1975) appears to be genetically much less variable between populations.

Mitochondrial DNA provides interesting information because of its maternal inheritance. *D. melanogaster* is moderately variable for mtDNA (Hale and Singh, 1987) but for *D. simulans*, a preliminary analysis (Baba-Aïssa and Solignac, 1984) of 13 lines suggests the occurrence of three geographic types with a low level of variability within each. This work has now been extended to 217 mitochondrial genomes from natural populations in various parts of the world, some of which have been studied with a wider range of restriction enzymes.

MATERIALS AND METHODS

Two hundred and seventeen isofemale lines were studied. They originate (table 1) from all continents and a number of oceanic islands. Between 1 and 21 lines from each natural population were studied.

Because of its maternal inheritance, all the flies of an isofemale line share the same mtDNA. MtDNA was extracted from unfertilized eggs using the quick preparation technique of Solignac *et al.*, 1986*a*. A large number of such eggs, on average 50,000, were used for extraction. The first extractions were performed from unfertilized eggs, laid by a number of virgin females. The egg production of virgins is low and irregular, and a more efficient method, using an interspecific cross, was worked out. Males of *D. mauritiana* (a sibling species of

Table 1 Geographic origin of the isofemale lines studied. N = number of lines. (Lines which were not fully analysed but only typed, in parentheses.) Mitochondrial genomes were assigned to the three main types (I, II and III) and subtypes (letters) as defined in fig. 1 by the structure of the non-coding region (the letter *x* is given to lines which were not assigned to a precise subtype). LH, length heteroplasmy; ARS, additional restriction site

		N	Types and subtypes	Remarks
Europe	Athens (Greece)	1	II*c*	
($N = 5$)	Sicilia (Italy)	1	II*c*	
	Antibes (France)	1	II*a*	
	Sevilla (Spain)	1	II*c*	
	Leeds (U.K.)	1	II*c*	*Hpa*II ARS
Africa	Alexandria (Egypt)	1	II*c*/*f*	LH
($N = 60$)	Aswan (Egypt)	1	II*c*	
North Africa	Nasr'allah (Tunisia)	1	II*a*	
($N = 5$)	Bizerte (Tunisia)	1	II*a*	
	Rabat (Morocco)	1	II*c*	
Tropical Africa)	Loua (Congo)	1	II*c*	
($N = 15$)	Brazzaville (Congo)	1	II*c*	
	Yaoundé (Cameroon)	1	II*d*	
	— (Ethiopia)	1	II*c*	
	Nairobi (Kenya)	1	II*c*/*e*	LH
	Mt Elgon (Kenya)	5	III*b*; 4II*c*	
	Mt Kenya (Kenya)	5	2II*c*; III*e*; III*q*; III*c*/*e*	LH
Southern Africa	Cape Town (S. Africa)	21 (15)	20II*c*, III*b*; 15II*x*	
($N = 40$)	Nysvley (S. Africa)	1	II*c*	
	Victoria Falls (Zimbabwe)	1	II*c*	
	Mwansa—(Tanzania)	2	II*b*; II*c*	
Middle East	Bagdad (Iraq)	8	5II*c*; III*q*; III*i*; III*c*/*f*	LH
($N = 17$)	Teheran (Iran)	9	8II*c*; III*b*	
America	Florida (U.S.A.)	1	II*c*	
($N = 10$)	South Amherst (U.S.A.)	1	II*a*	
	Archibald (U.S.A.)	1	II*c*	
	Carmel (U.S.A.)	1	II*c*	
	Mapimi (Mexico)	3	II*c*	
	— (Panama)	1	II*c*	
	Cayenne (French Guyana)	1	II*c*	
	Porto Allegro (Brazil)	1	II*c*	
Australia	Sydney	1	II*c*	
($N = 2$)	Melbourne	1	II*c*	
Indian Ocean region	Mont-d'Ambre (Madagascar)	1	III*a*	
($N = 84$)	Antananarivo (Madagascar)	15	8III*a*; 7II*h*	
	Réunion Island	3 (37)	3III*a*; 35III*x*; 2II*x*	
	Comoro Islands	5	II*c*	
	Mahé (Seychelles)	16 (21)	8I*a*; 22I*b*; 3I*d*; 3I*a*/*b*; 1I*b*/*d*	*Sac*I ARS LH
Pacific Ocean islands	Ogasawara (Japan)	1	II*c*	
	Hawaii (U.S.A.)	7	6I*b*; 1I*c*	
	Moorea (French Polynesia)	11	9I*b*; 1I*e*; 1Ib/d	LH
	Nouméa (New Caledonia)	6	6I*b*	*Sac*I ARS

D. simulans) mate readily with *D. simulans* females, producing sterile F1 males but fully fertile females (David *et al.*, 1974, 1976). Keeping the F1 flies together provides many unfertilized eggs with *D. simulans* cytoplasm.

The extracted DNA was digested with restriction enzymes. In our preparations, contaminant nuclear DNA amounts to about half of the total (Goldring and Peacock, 1977). Because of its complexity, this is digested into numerous fragments

of diverse sizes which, after electrophoresis, produce a slight smear on the gels. By contrast, mtDNA is cut into a few, well defined fragments which are clearly identified.

Ten restriction enzymes were used to study 144 lines. They are *Acc*I, *Bgl*II, *Eco*RI, *Hae*III, *Hin*dIII, *Hpa*I, *Hpa*II, *Pvu*II, *Sac*I, *Xba*I. Five additional enzymes (*Ava*I, *Ava*II, *Bcl*I, *Cla*I and *Eco*RV) were used on a smaller subsample of 30 isofemale lines. The remaining 73 lines, originating from South Africa (15 lines), Réunion Island (37 lines) and the Seychelles (21 lines) were analysed with only a few diagnostic enzymes in order to identify their mitochondrial type (see results section).

Restriction fragments were separated by electrophoresis either on 1 per cent agarose or on 5 per cent polyacrylamide gels. After staining with ethidium bromide, each gel was photographed under UV light. For *Acc*I digestions, restriction fragments were radioactively end-labelled and the restriction fragments revealed by autoradiography.

Pairwise nucleotide distances π_{ij} were calculated between all variants (types and subtypes) using the formulae of Nei and Li (1979). Nucleotide diversity π was estimated, using the weighted average of pairwise nucleotide distances.

RESULTS

1. The three mitochondrial DNA types

The mitochondrial genomes of the 144 lines studied here with 10 or 15 enzymes (and by inference those of the 73 additional lines) can be assigned to three geographically distinct main types, *si*I, *si*II and *si*III. Their restriction maps have been published by Solignac and Monnerot (1986) and Solignac *et al.* (1986a). Six restriction enzymes, *Acc*I, *Cla*I, *Eco*RI, *Eco*RV, *Hpa*I and *Hpa*II are diagnostic. They provide characteristic electrophoretic profiles so that any mtDNA digested with one of these enzymes can be unambiguously assigned to one of the types.

In the coding region of the mtDNA, the types differ by 10 to 15 restriction sites among the 40 or so identified in the present study. Within a given type, variability is absent or is restricted to a single site. In the A+T-rich region variations are observed between the three types and also within two of them, allowing the definition of subtypes (fig. 1). This region comprises a domain harbouring a sequence of 470 bp which is repeated from two to four times in direct tandem. Each repeated unit typically shows an *Hpa*I and an *Acc*I site, mapped very close together. One of these two sites may be absent in one, several or even all of these units. The repeated region is flanked by domains whose lengths are also variable (see Solignac *et al.*, 1986*b*).

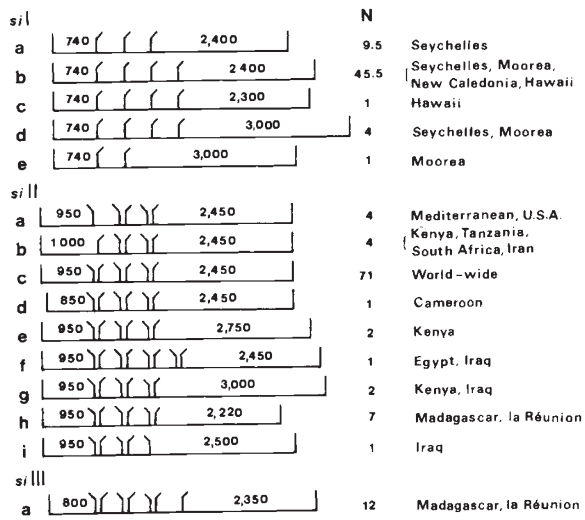

Figure 1 Structure of the A+T-rich, non-coding region in three types of mitochondrial genomes. The region shown, slightly larger than the control region itself, is flanked on the left by a *Hin*dIII restriction site within the ND2 gene and, on the right, by a *Tha*I site within the small subunit of the ribosomal RNA. The *Hpa*I sites are indicated by left-leaning sticks and *Acc*I sites by right-leaning sticks.

These sites are included in a repeated unit of 470 bp. The domains on the left and right of the repeated sequences are variable in length. Within each main type (I, II, III) structural subtypes are designated by letters. For each of them the number of lines (N) observed and the geographic origin are indicated. The size of double digest fragments is indicated in base pairs.

2. The *si*I type

All the flies originating from the following populations: Seychelles (37 lines), New Caledonia (6 lines), Moorea (11 lines) and Hawaii (7 lines) belong to the *si*I type. No other type was detected on these islands and no *si*I mtDNA was found in the other populations studied (table 1).

In the coding region, only one polymorphic site was observed. This corresponds to an extra *Sac*I site found in two lines from New Caledonia and one from the Seychelles. The 13 lines analysed with five additional enxymes did not show any further variant.

Length variations in the A+T-rich region (fig. 1) allowed the definition of five subtypes (*a* to *e*). There is variation in the number of repeated sequences (2 to 4 units) and in the length of the domain located to the right of these sequences. *si*I*b* is always the commonest subtype. The other subtypes are each present only on one island (except *si*I*d*, which is found on Mahé and Moorea).

Five out of 61 lines were heteroplasmic for genome length: three lines had the structure *a*/*b* and two the structure *b*/*d* (table 1; fig. 1).

3. *The si*II *type*

This widespread genome was found in 109 lines from Africa, Europe, America, the Middle East, Australia and Japan where all lines belonged to the *si*II type. It was also found in Madagascar (7 out of 16 lines) and in Réunion (2 out of 40). Type II is hence almost a worldwide genome and may well comprise more than 95 per cent of the individuals and populations of this cosmopolitan species (fig. 2).

Only one polymorphic site was detected in the coding region of the 92 lines analysed with a complete set of 10 enzymes: it is an additional *Hpa*II site found, in an English line from Leeds (the same variant was found in a Italian line by Nigro, 1988). Five additional enzymes used on 15 lines failed to evidence any extra variation.

The non coding region (fig. 1) was more variable, as its length varies with the number of repeated units (3 or 4) and with the size of domains to the right or left of the repeated region. There is a polymorphism in the repeated units, for one *Hpa*I and two *Acc*I sites. Nine subtypes, *a* to *i*, can be distinguished. The *c* subtype is the most common (72 per cent). Among 92 genomes, four cases of length heteroplasmy were observed: two (Egypt and Iraq) with the *c*/*f* structure and two (Kenya) with *c*/*e*.

4. *The si*III *type*

This is found in Réunion (38 lines out of 40) and in Madagascar (nine out of 16). Ten lines were studied with 10 enzymes and two with 15 enzymes

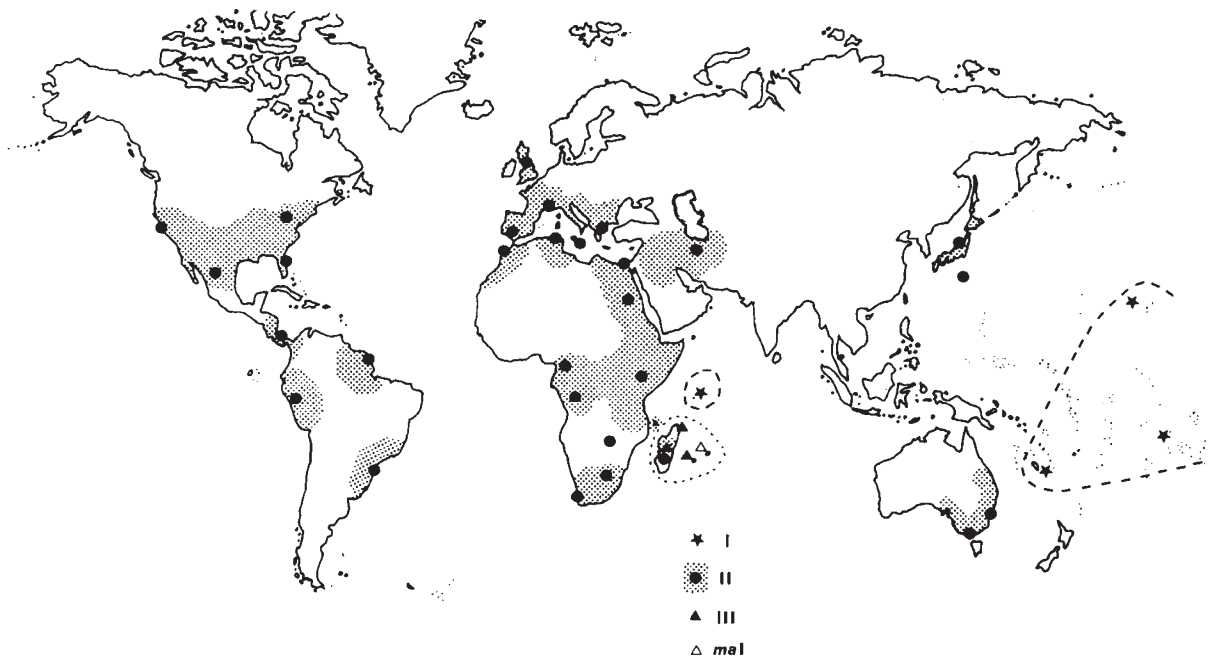

Figure 2 Distribution of mtDNA types in *D. simulans*. Type *si*I is restricted to the Seychelles, New Caledonia, Polynesia and Hawaii. Type *si*III is found only in Madagascar and Réunion but occurs also in the sibling species *D. mauritiana* in Mauritius. Type *Si*II is worldwide and, around each locality, a dotted area shows the presumed extension of the population. Localities are shown in table 1 and results from other authors have been included: North Carolina and Japan (Shah and Langley, 1979*a*); California and Peru (Fauron and Wolstenholme, 1980*b*); Italy, Netherland, Spain and Morocco (Nigro, 1988).

(three from Réunion and nine from Madagascar) but no variant was detected either for the restriction sites or for the genome length. The non coding region always harbours four repeated sequences (fig. 1).

This *si*III type is identical, with respect to restriction sites and structure of the A+T-rich region, to the long *ma*I genome which exists in *D. mauritiana* (Solignac *et al.*, 1983).

5. Polymorphism and nucleotide distances

Data on a polymorphism (excluding the 73 lines only assigned to a given type and results obtained with the five additional enzymes) are summarized in table 2. Polymorphism is calculated for the coding region, the non-coding region and the complete genome. In the A+T-rich region, the alignment of *Hpa*I and *Acc*I sites cannot be directly done when the number of repeated units are different from the commonest genome that carries three repeated sequences (fig. 1). For this reason, in order to allow alignment, one unit has been taken off in the genomes which have four units (*si*I*b*, *c*, *d*, *si*II*f* and *si*III*a*) and the single genome where only two units are found (*si*I*e*) has been considered as possessing an additional (fictitious) unit with one *Hpa*I site. In table 2, the three main mitochondrial types are subdivided into groups which take account of site variations in the subtypes. Within each type, the distances between subtypes are extremely low. As expected, distances

Table 2 Matrices of restriction site numbers (on and above diagonal) and of nucleotide distances (below). Mitochondrial subtypes are distinguished, within each type, according to variation in the non coding region (see fig. 2). In *si*I type, a subtype *Sac*I+ is distinguished by an extra restriction site (three lines). In *si*III*c* one line exhibited an additional *Hpa*II site. Numbers of lines are given for each type. In each box the first row corresponds to the non coding region, the second row to the coding region, the third to the total genome.

On the diagonal: number of restriction sites observed with 4-, 5- and 6-cutter enzymes.

Above the diagonal: number of restriction sites in common between types and subtypes, using the three classes of enzymes.

Below the diagonal: nucleotide distances (%) calculated according to Nei and Li (1979).

		I 40		II*a* 4	II*b* 4	II*c–h* 83		II*i* 1	III*a* 12
		*Sac*I^{-1} 37	*Sac*I^{+} 3			*Hpa*II^{-} 82	*Hpa*II^{+} 1		
I	*Sac*I^{-}	0+3+0 6+6+28 6+9+28	0+3+0 6+6+28 6+9+28	0+2+0 5+3+24 5+5+24	0+3+0 5+3+24 5+6+24	0+3+0 5+3+24 5+6+24	0+3+0 5+3+24 5+5+24	0+2+0 5+3+24 5+5+24	0+3+0 4+4+26 4+7+26
	*Sac*I^{+}	0 0·23 0·21	0+3+0 6+6+29 6+9+29	0+2+0 5+5+24 5+5+24	0+3+0 5+3+24 5+6+24	0+3+0 5+3+24 5+6+24	0+3+0 5+3+24 5+6+24	0+2+0 5+3+24 5+5+24	0+3+0 4+4+26 4+7+26
II*a*		11·55 3·87 4·46	11·55 4·08 4·67	0+2+3 6+3+30 6+5+33	0+2+2 6+3+30 6+5+32	0+2+3 6+3+30 6+5+33	0+2+3 6+3+30 6+5+33	0+1+3 6+3+30 6+4+33	0+2+2 4+3+28 4+5+30
II*b*		4·79 3·87 3·92	4·79 4·08 4·12	3·72 0 0·43	0+3+2 6+3+30 6+6+32	0−3+2 6+3+30 6+6+32	0+3+2 6+3+30 6+6+32	0+2+2 6+3+30 6+5+32	0+3+1 4+3+28 4+6+29
II*c–h*	*Hpa*II^{-}	4·76 3·87 4·12	6·76 4·08 4·33	1·59 0 0·23	1·59 0 0·20	0+3+3 6+3+30 6+6+33	0+3+3 6+3+30 6+6+33	0+2+3 6+3+30 6+5+33	0+3+2 4+3+28 4+5+33
	*Hpa*II^{+}	6·76 4·12 4·34	6·76 4·32 4·54	1·59 0·25 0·44	1·59 0·25 0·42	0 0·25 0·22	0+3+3 7+3+30 7+6+33	0+2+3 6+3+30 6+5+33	0+3+2 4+3+28 6+5+33
II*i*		11·55 3·87 4·46	3·72 4·08 4·64	3·72 0 0·53	1·59 0 0·43	1·59 0 0·23	1·59 0·25 0·44	0+2+3 6+3+30 6+5+33	0+2+2 4+3+28 4+5+30
III*a*		4·79 3·57 3·67	4·79 3·74 3·84	3·72 2·90 3·00	3·72 2·90 2·98	1·59 2·90 2·75	1·59 3·34 3·14	3·72 2·90 3·00	0+3+2 7+4+30 7+7+32

between types are much higher, *si*II and *si*III being closer between them than with *si*I.

The nucleotide diversity, π, is given in table 3. This has been calculated for some natural populations (*e.g.*, Seychelles, Cape Town) when the sample size was sufficient, for distinct geographic regions (*e.g.*, Europe, Africa, etc.), for each of the three types, and for the whole species. Within each type, the diversity is very low, on the average 0·030 per cent. It is much higher (1·98 per cent) for the whole species.

Table 3 Nucleotide diversity (π of Nei and Tajima, 1981) in the mitochondrial genome of *D. simulans*. The results are presented according to the country of origin (when a sufficient number of lines were available), to larger geographic regions, to mitochondrial types or to the whole species. Values are calculated for the coding region and the total genome, and are expressed in percentages. The number of lines is given in parentheses

	Coding region	Total genome
Country of origin		
Seychelles (16)	0·029	0·026
Southern Africa (25)	0·000	0·031
Madagascar (16)	1·523	1·444
Geographic regions		
Europe (5)	0·100	0·179
Africa (45)	0·000	0·045
Middle East (17)	0·000	0·051
America (10)	0·000	0·046
mt DNA types		
type I (40)	0·033	0·030
type II (92)	0·006	0·046
type III (12)	0·000	0·000
Whole species (144)	1·873	1·973

DISCUSSION

There are three main, well differentiated and mostly allopatric mtDNA types in *D. simulans* with a very low genetic polymorphism within each of them. These can be related to the past evolutionary history of the species and also to its population dynamics and recent colonizing history.

Each mitochondrial type occupies a distinct geographic area. Type II, found in almost all temperate and tropical continental areas and in many islands, is almost worldwide. The two other types are only found on a few islands: type *si*III only on Madagascar and its neighbour, Réunion and *si*I is from four areas separated by up to 17,000 km. Only in Madagascar and Réunion are two types (II and III) found in sympatry.

The domestic status of *D. simulans* suggests that the occurrence of two types on these islands may be a consequence of recent introductions, with *si*III (endemic to this region), as the native population and *si*II (represented by the *h* endemic subtype of unknown origin), being a recent colonizer. The three groups of populations are three cytoplasmic geographic races within *D. simulans*. Hybridization experiments between races do not show any incipient isolation (Lachaise *et al.*, 1986 and unpublished).

In the coding region, intratype variants are very scarce: an additional *Sac*I site was encountered in three individuals, belonging to the type I and an extra *Hpa*II site was found in a type II strain from Leeds. Variation is more common in the A+T-rich control region (fig. 1). The number of repeated units ranges from 2 to 4, and the length of the domains at the left or the right of these units also shows some variability. Within the repeated units, there is a polymorphism of some *Hpa*I and *Acc*I sites in *si*II clones: an observation which agrees with previous work on *Drosophila* (Shah and Langley, 1979b; Zakour and Bultmann, 1979; Fauron and Wolstenholme, 1980*a*; Clary and Wolstenholme, 1987).

Five-and-a-half per cent of variants were found in an heteroplasmic state. In the other *Drosophila* species, possessing a long mitochondrial genome, the frequency of heteroplasmic individuals is 18 per cent in *D. mauritiana* (Solignac *et al.*, 1984 and 1986*a*, *b*), 12 per cent in *D. sechellia* (Solignac *et al.*, 1986*b* and unpublished) and 17 per cent in *D. melanogaster* (Hale and Singh, 1986).

Genetic variability in the *D. simulans* mtDNA ($\pi = 1{\cdot}98$ per cent) (table 3) is within the range of values found in vertebrates (where the nucleotide diversity generally ranges between a few per thousand and a few per cent: Avise and Lansman, 1983; Boursot and Bonhomme, 1986; Avise *et al.*, 1987) and in genus *Drosophila*: 1·1 per cent and 0·45 per cent for *D. subobscura* in the Old and New Worlds respectively (Latorre *et al.*, 1986); 1·1 per cent for *D. heteroneura* and *D. silvestris* in Hawaii (from data of DeSalle *et al.*, 1986, and formula 12 of Ewens *et al.*, 1981), and 0·2 per cent in *D. melanogaster* (Hale and Singh, 1987).

However, the nucleotide diversity in *D. simulans* is almost entirely due to the existence of the inter-racial variability as intra-racial variability, for the coding regions is only 0·033, 0·006 and 0·000 per cent for *si* I, *si*II and *si*III.

The world-wide *si*II race hence has a variability two or three levels of magnitude lower than the Hawaiian *Drosophila* which have a far smaller

population size over a tiny geographic area. Some efficient process must have reduced mitochondrial variability in *D. simulans*.

Two stochastic processes may be involved: a severe bottleneck of short duration (founder effect) or long lasting genetic drift in a small population. We previously (Baba-Aïssa and Solignac, 1984) favoured the founder effect hypothesis for *D. simulans* as did Brown (1980) for human beings. This hypothesis has been criticized by several authors (Avise *et al.*, 1984; Latorre *et al.*, 1986) who defend the possibility of a chronic genetic drift. These processes cannot easily be distinguished by just considering the mitochondrial genome.

Contrary to earlier estimates, recent studies of intrapopulational enzymatic variability of *D. simulans* have shown almost as high a heterozygosity as that of *D. melanogaster* (Hyytia *et al.*, 1985; Choudhary and Singh, 1987; Cariou, personal communication). The low mitochondrial variability within each race, associated with a normal heterozygosity of nuclear genes, is an argument in favour of founder event (see Wilson *et al.*, 1985). The genetic drift necessary to reduce the mtDNA variability to its current level would be severe; for *si*II, the long term effective size would have to be around 1000 females (if we assume that π is an estimate of 4 Nμ, and accept, with DeSalle *et al.* (1987), that the mutation rate, $\mu = 10^{-8}$ for *Drosophila* mtDNA). However, the genetic diversity of nuclear genes ($H = 0{\cdot}09$, Choudhary and Singh, 1987) leads to an estimate of population size 100 times higher, according to the formula of Kimura and Crow (1964).

D. simulans probably originated in Eastern Africa (mainland or neighbouring Indian Oceanic islands) an hypothesis sustained by faunistic arguments (Lachaise *et al.*, 1988), the presence of three mitochondrial genomes here and the two insular and closely related endemics *D. mauritiana* and *D. sechellia* (Solignac and Monnerot, 1986). *D. simulans* probably achieved its present world-wide distribution through human transport (David and Tsacas, 1981; Parsons, 1983; Lachaise *et al.*, 1988). The recency of such an expansion is attested to by its mitochondrial monomorphism over large areas and by the great genetic similarities of the nuclear genomes of remote populations (Hyytia *et al.*, 1985; Choudhary and Singh, 1987).

It is not clear whether man has propagated individuals from three monomorphic ancestral races, or if the different mitochondrial types all originated in a single ancestral polymorphic population. Founder effects are compatible with either hypothesis, but a dramatic genetic drift is unlikely to have preserved the three very different—and very ancient—mitochondrial types so that this second hypothesis seems unlikely.

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