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Dubious maternal inheritance of mitochondrial DNA in *D. simulans* and evolution of *D. mauritiana*

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Summary

Within-line heterogeneity has been found in the mitochondrial DNA (mtDNA) in two isofemale lines of *D. simulans*. The co-existing types, S and M, were typical of the mtDNA in *D. simulans* and in *D. mauritiana*, respectively, their nucleotide divergence per site being *ca.* 2·1%. Segregation analysis confirmed that some individuals in these lines were heteroplasmic and suggested incomplete maternal inheritance of mtDNA in *Drosophila*. Examination of other lines of *D. simulans* revealed that the M type of *D. mauritiana* occurs at 71% in Réunion, 38% in Madagascar and 0% in Kenya. This finding and interspecific sequence comparisons of both M types indicate that *D. mauritiana* diverged from *D. simulans* probably less than 240000 years ago.

1. Introduction

Mitochondrial DNA (mtDNA) in animals is represented by numerous copies within a cell (Gillham, 1978; Beale & Knowles, 1978) and is maternally transmitted (Giles *et al.* 1980; Lansman, Avise & Huettel, 1983). The cellular mechanisms, though poorly understood, that regulate the replication and degradation of mtDNA and the distribution to daughter cells at each cell division ensure that when a variant occurs, the copy number fluctuates with time and eventually reaches 0% (loss) or 100% (fixation) in a germ-cell lineage (Hauswirth & Lapis, 1982; Birky, 1978; Solignac *et al.* 1984). However, loss or fixation occurs rather rapidly and independently in different individuals, and under strictly maternal inheritance of mtDNA any variant arising in a particular individual can spread only over individuals that are maternally identical by descent. Therefore heteroplasmy, coexistence of more than one type of mtDNA within a cell or individual, is rarely found in natural populations unless variants arise frequently and/or the copy number is large enough to maintain the heteroplasmic state for a number of generations. Heteroplasmy observed to date is mainly on the length variation (Solignac, Monnerot & Mounolou, 1983; Harrison, Rand & Wheeler, 1985; Boursot, Yonekawa & Bonhomme, 1987) due presumably to the high rate of occurrence of such variation. By contrast, the heteroplasmy reported here contained a mtDNA of a different species, ruling out the possibility that it appeared independently by mutation. The evolutionary implication should be quite different from that reported previously.

We report also that one type of mtDNA is shared by *D. simulans* and *D. mauritiana*. There are two possible causes for this. One is due to old polymorphism, which is a remnant of the fact that orthologous genes once existed as alleles in the ancestral population common to the extant species under study. The divergence (coalescence) time of alleles may be sufficiently long (Kingman, 1982) so that the divergence of orthologous genes can be much older than the species divergence. The other is due to introgression or secondary hybridization in which orthologous genes in one species are transmitted to another over species boundaries (e.g. Ferris *et al.* 1983). In this case, species undergoing introgression may acquire quite distinct alleles from their own. The finding that one type of mtDNA is extensively shared by the two sibling species sheds new light on the evolution of *D. mauritiana*.

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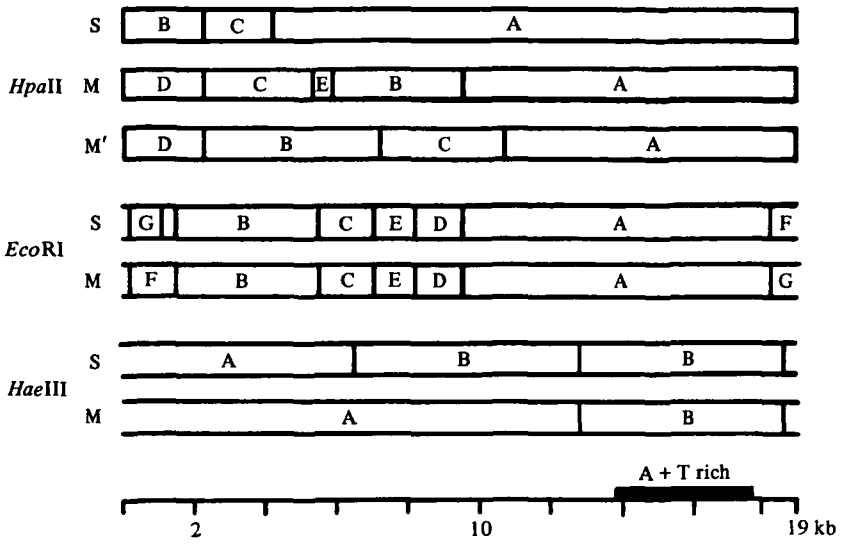

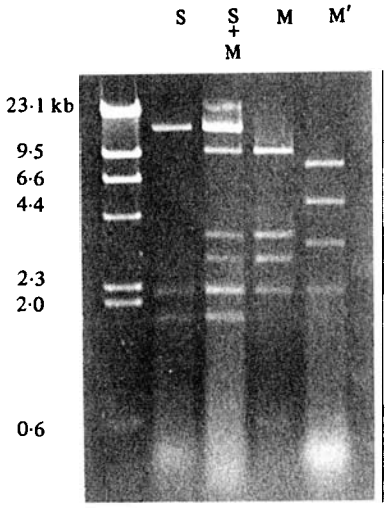

Fig. 1. Restriction maps of S and M types of mtDNA with three restriction enzymes, and digest patterns of S, M, M′ (*ma*II in Solignac *et al.* 1986) and S+M (heteroplasmy) with *Hpa*II. Mitochondria were isolated from about 300 adult flies by differential centrifugation and purified through discontinuous sucrose gradient centrifugation. The SDS-phenol treatment of these mitochondria yielded about 1 μg mtDNA. The fragments cut by a restriction enzyme were separated by electrophoresis on 1·0% or 0·8% agarose gel. The gel was then stained with ethidium bromide and photographed with Polaroid camera under UV-light. *Hin*dIII/λ phage DNA digest, as a molecular weight standard, is shown on the left-most lane. The circular genome is drawn as if linear, one of the *Hpa*II sites common to S and M being both ends. The capital letters in each bar show relative fragment sizes in the decreasing order.

2. Materials and results

Among 48 isofemale lines examined, 17 are of *D. simulans* from Réunion, 8 from Madagascar, 10 from Kenya, and 13 of *D. mauritiana* endemic to Mauritius. All these lines were established in 1979 and had been maintained in mass culture for six years until the present study started. *D. simulans* and *D. mauritiana* are 2 of the 8 species of the *melanogaster* subgroup and are very similar to each other in chromosome banding patterns (Lemeunier & Ashburner, 1976), protein polymorphisms (Gonzáles *et al.* 1982) and DNA sequences at the alcohol dehydrogenase (*Adh*) locus (Cohn, Thompson & Moore, 1984; Bodmer & Ashburner, 1984; Coyne & Kreitman, 1986). *D. mauritiana* is thought to have arisen from a population of proto-*simulans* colonizing Mauritius 180 km northeast of Réunion. The cross between *D. simulans* females and *D. mauritiana* males produces sterile male and fertile female hybrids, but the reciprocal cross is difficult (David *et al.* 1976; Coyne & Charlesworth, 1986).

mtDNA extracted from each isofemale line (see the legend in Fig. 1 for the method) was digested by restriction enzymes. The restriction maps of *Hae*III, *Eco*RI and *Hpa*II for ten lines of *D. simulans* from Kenya were identical to each other and so were all but one line of *D. mauritiana*. However, the two species differed at six recognition sites, out of which four are distinguished by *Hpa*II (Fig. 1). Thus *Hpa*II could be used most reliably to diagnose the species-specific types of mtDNA. These types are designated as S and M, which are identical to *si*II and *ma*I, respectively, in Solignac, Monnerot & Mounolou (1986).

Table 1. *Number of isofemale lines classified by mtDNA genotypes*

	S	M(M′)	S+M
D. simulans			
Kenya (10)	10	0 (0)	0
Madagascar (8)	5	3 (0)	0
Réunion (17)	3	12 (0)	2
D. mauritiana			
Mauritius (13)	0	12 (1)	0

S and M, representative types of mtDNA in *D. simulans* and that in *D. mauritiana*; M′, a minor type in *D. mauritiana* identical to *ma*II in Solignac *et al.* (1986); S+M, heterogeneous lines that possess both S and M.

When 17 lines from Réunion and eight from Madagascar were digested by *Hpa*II, striking results emerged (Fig. 1 and Table 1). Out of 25 lines, two from Réunion, denoted by SI201 and SI203, were heterogeneous for S and M types. Moreover 12 lines from Réunion and three from Madagascar had only the M type. Analysis of *Eco*RI and *Hae*III fragments also showed these results.

The heterogeneity of SI201 and SI203 implies that the single impregnated females that originated these lines must have been heteroplasmic with respect to S and M. To see whether heteroplasmy really existed in natural populations, we constructed 44 sublines by

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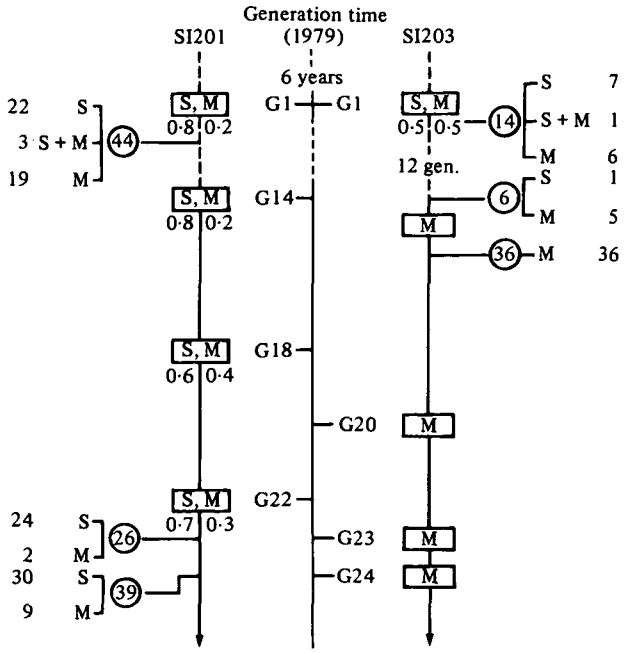

Fig. 2. Relative frequencies of S and M in heterogeneous lines SI201 and SI203, and segregation patterns of homoplasmy. S and M in a box show that a line in mass culture is heterogeneous, and their relative frequencies measured from densitometric profile are given under boxes. At some generations, subisofemale lines were constructed to examine the existence of heteroplasmy and segregation of homoplasmic individuals. Circled are the numbers of inseminated females isolated for subline construction. Each female was set in a culture vial to produce F_1. F_1 were further transferred into new vials to obtain F_2 from which mtDNA was extracted as described in Fig. 1. Mitochondrial genotypes of isolated females and their numbers are shown in side branches. A homogeneous line implies the non-existence of heteroplasmy, but a heterogeneous line does not necessarily imply the existence of heteroplasmy.

randomly sampling inseminated females from SI201, and 14 sublines from SI203. The results of *Hpa*II assay for these sublines is depicted in Fig. 2, indicating that SI201 and SI203 were not only heterogeneous but had maintained heteroplasmic individuals until the time of subline sampling. Thus heteroplasmy in these lines had persisted over six years. The same assay on later generations, however, showed that SI203 is homogeneous for M and that although SI201 is still heterogeneous, heteroplasmy might disappear. The number of sublines homogeneous for M at each subline sampling was roughly proportional to the frequency of M in SI201 and SI203 at that time. This indicates that S and M are functionally equivalent and their frequency changes within a cell are subject to pure 'random drift'. Further segregation analysis of heterogeneous sublines is under way.

To further confirm that M_s, the M type in *D. simulans*, is the same as M_m, the M type in *D. mauritiana* (by definition $M_m = M$), a common region 975 bp long, which encodes three tRNAs, the 3′ portion of NADH dehydrogenase subunit 2 and 5′ portion of cytochrome oxidase subunit 1, was sequenced and compared by the method in Satta *et al.* (1987, data not shown). No nucleotide differences were found whereas S and M differ at 19 nucleotide sites in this region (Satta *et al.* 1987). Thus it is highly improbable that M_s was newly derived from S since the divergence of M_m. The preponderance of M_s in Réunion and Madagascar also support this view and suggests that the age of M_s must be fairly old.

These findings are not easily accounted for by our current knowledge on transmission genetics of mtDNA and evolutionary relatedness between *D. simulans* and *D. mauritiana* so that we examined *D. simulans* of M type to find if it is the same as all other *D. simulans* in morphological traits and mating behaviour. The only consistent morphological difference between *D. simulans* and *D. mauritiana* is thought to be the shape of the posterior process of the male genital arch. The process of all *D. simulans* of M type, descended from homo- or heteroplasmy, was indeed large and helmet-shaped, which is very different from the slender and finger-like process of *D. mauritiana*. Furthermore, to see if there is premating isolation between these two species, a mating experiment was carried out. All *D. simulans*, irrespective of their ancestry and mitochondrial genotype, showed the same mating behaviour: *D. simulans* females could mate successfully with *D. mauritiana* males, but the reciprocal cross could not happen (Table 2). These results suggest that the nuclear genome of all the lines of *D. simulans* examined is equivalent to that of authentic *D. simulans*, although more direct evidence would be preferable and a closer look at specific nuclear loci at the DNA level would be very informative.

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Table 2. *Percentage of successful matings (number of crosses) within and between isofemale lines of* D. simulans *and* D. mauritiana

	Males						
Females	1	2	3	4	5	6	7
D. simulans							
1 Kenya (S)	95·7 (47)	—	—	—	—	—	95·8 (48)
2 Madagascar (S)	—	77·8 (45)	—	—	—	—	89·4 (47)
3 Réunion (S)	—	—	87·8 (41)	—	—	—	62·8 (43)
4 Réunion (M)	—	—	—	96·0 (50)	—	—	95·8 (49)
5 Réunion (S*)	—	—	—	—	100 (41)	95·3 (43)	90·7 (43)
6 Réunion (M*)	—	—	—	—	88·4 (43)	91·1 (45)	87·5 (40)
D. mauritiana							
7 Mauritius (M)	0·0 (41)	0·0 (41)	0·0 (41)	0·0 (44)	0·0 (41)	0·0 (41)	89·4 (46)

The percentage of successful matings was estimated as the ratio of the number of crosses that produced F_1 progeny to the total number of crosses, in each of which one female and two males were placed in a vial for 5 days. S and M, homogeneous lines with mitochondrial genotypes S and M as in Table 1; S* and M*, homogeneous sublines stemmed from heterogeneous line SI201 and fixed by S and M, respectively.

3. Discussion

Two hypotheses, though not mutually exclusive, are examined in the light of the present results. Firstly, we consider the hypothesis that M_s and M_m shared by the two sibling species are remnants of an old polymorphism. If M_s and M_m had occurred in the ancestral population from which *D. mauritiana* branched off, the coexistence can be explained by their fortuitous distribution to descendant lineages at speciation and their independent evolution as orthologous mtDNAs from that time on. As shown for *Adh* F and S alleles in *D. melanogaster* (Kreitman, 1983; Stephens & Nei, 1985), old polymorphism is not a rare phenomenon, and its recognition is very important in molecular taxonomical studies particularly when species compared are closely related (Takahata & Nei, 1985).

Under the above hypothesis and if we assume that reproductive isolation became complete t_s years ago, it is possible to estimate an upper bound of the divergence time (t_s) of *D. simulans* and *D. mauritiana* from the divergence time (t) of M_s and M_m. Noting that the probability that no change occurs at n sites since the separation of M_s and M_m is approximately given by $\exp(-2knt)$ in which k is the substitution rate per site per year, we obtain $t = -\log_e(0{\cdot}01)/(2kn) = 2{\cdot}3/kn$ for 99% confidence. The calibration of substitution rates in *Drosophila* is difficult, but if $k = 10^{-8}$ (see Satta *et al.* 1987 as a conservative estimate) and for $n = 975$, t is unlikely to be longer than 240000 years. With the same value of k, the divergence time of S and M amounts to about one million years, which is roughly the same as the estimate of species divergence time (t_s) of *D. mauritiana* from *D. simulans* (Stephens & Nei, 1985). It is noteworthy, however, that the standard error on t_s was so large that a more recent or ancient colonization of proto-*simulans* could not be ruled out. Thus in the absence of interspecific gene flow (see below), we conclude that the colonization must have occurred very recently. This conclusion is also consistent with the high frequency of M (Table 1) and an independent finding of M in Madagascar (Bab-Aïssa & Solignac, 1984; Solignac *et al.* 1986). However, *D. mauritiana* has derived from proto-*simulans* which possessed apparently not only the M type. In fact, it was found that *D. mauritiana* has another distinct type of mtDNA (M′ in Table 1) whose nucleotide divergence from M is *ca.* 1·9% per site (Solignac *et al.* 1986). This hypothesis predicts that S, M and M′ diverged around one million years ago, four times earlier than the divergence of M_s and M_m and therefore prior to speciation of *D. mauritiana*, and that *D. simulans* may also carry M′.

Secondly, we consider the hypothesis that M_s and M_m has recently been transmitted across species boundaries. Interspecific transfer of mtDNA has been reported in mice (Ferris *et al.* 1983), frogs (Spolsky & Uzzell, 1984) and *Drosophila* (Powell, 1983). Extensive mitochondrial gene flow, if Table 1 is understood as such, without appreciable nuclear gene flow (Table 2) can be accounted for if only the nuclear genome is involved in reproductive barrier. Selection against hybrids can then prevent introgression of the nuclear genome while allowing introgression of mtDNA (Takahata & Slatkin, 1984; Takahata, 1985). Although Madagascar, Réunion and Mauritius are geographically isolated, the prevalence of recent commercial traffic among these islands may have promoted mitochondrial gene flow. Introgression might have occurred from *D. mauritiana* to *D. simulans*, or vice versa. However, there is a difficulty in either direction. In the former, pre-mating isolation between *D. mauritiana* females and *D. simulans* males is well established (David *et al.* 1976) so that the levels of mitochondrial gene flow from *D. mauritiana* might be too low to explain the high frequency of M in

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Réunion and Madagascar. In the latter (e.g. Solignac & Monnerot, 1986), we could have found S in *D. mauritiana* in addition to M because S is a predominant type of mtDNA in Madagascar and Africa (Table 1) and there is no evidence that gene flow occurs only between Réunion and Mauritius. Thus this hypothesis appears less likely than the first one.

To further substantiate the above conclusions, it is important to make more extensive sequence analysis between M_s and M_m. If a significant amount of differences is observed, the first hypothesis would be confirmed, but if the differences turn out to be extremely small, the second hypothesis cannot be excluded. For instance, if $k = 10^{-8}$ and $t = 240000$, the probability of no change between two orthologous sequences becomes 0·00006 for $n = 2000$, 0·0000006 for $n = 3000$ and so forth. The extremely small probability implies the much shorter divergence time of M_s and M_m, suggesting the possibility of recent introgression.

To interpret heteroplasmic lines SI201 and SI203, both hypotheses must assume that maternal inheritance is not complete or that mtDNA can be horizontally transmitted between individuals. Little is known about horizontal transfer of mtDNA so that we tentatively dismiss such a possibility. Under this situation, incomplete maternal inheritance seems the only likely mechanism that can account for the heteroplasmy reported here.

To fully elucidate the problems concerning transmission genetics of mtDNA and evolutionary relatedness of *D. simulans* and *D. mauritiana*, we need to assess the extent of paternal contribution of mtDNA, the rate of segregating homoplasmy and the nucleotide divergence at several nuclear loci. Extensive survey of natural populations is also required to confirm that these species might have diverged more recently than previously thought.

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