Frequencies of Mitochondrial DNA Haplotypes in Laboratory Cage Populations of the Mosquito, *Aedes albopictus*

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ABSTRACT

A laboratory cage experiment was undertaken to study changes over time in the frequencies of two mitochondrial DNA (mtDNA) haplotypes in the mosquito, *Aedes albopictus*, under two conditions: bidirectionally compatible matings and unidirectionally incompatible matings. Frequencies were monitored for 10 generations in three replicate cages for each of the two conditions above. In cages with bidirectionally compatible strains, changes in haplotype frequencies were nondirectional and neither haplotype increased in frequency. Statistical analysis of relative proportions of the two haplotypes in each generation indicated that the magnitude of the observed fluctuations could be expected under an assumption of random genetic drift alone. In cages with unidirectionally incompatible matings, mtDNA of females that lay inviable eggs upon mating with males of another strain, decreased significantly in the F_1 generation and was completely replaced in the F_2 generation.

ANIMAL mitochondrial DNA (mtDNA) has been used extensively in studies of evolutionary and population genetics in recent years [see AVISE *et al.* (1987) and WILSON *et al.* (1985) for reviews]. Because of its maternal inheritance, lack of recombination and rapid rate of evolution relative to nuclear DNA in most animals, it is a useful tool for studying spatial subdivision of populations, gene flow across hybrid zones and reconstruction of phylogeny. Implicit in the use of mtDNA for such purposes is the assumption that minor restriction site or DNA sequence differences among haplotypes are selectively neutral.

MACRAE and ANDERSON (1988), in a study of two strains of *Drosophila pseudoobscura* (Apple Hill, California, and Bogotá, Colombia), suggested that mtDNA haplotypes are not selectively neutral but are under selection. They found that the frequency of Bogotá mtDNA increased by 46% in three generations in one of four replicate cages and reached an apparent equilibrium frequency. MACRAE and ANDERSON (1988) invoked "sporadic selection" to explain the results. If correct, and found to be a general phenomenon, their results have an important bearing on the use of mtDNA for evolutionary and population genetic studies.

MACRAE and ANDERSON's experimental design, choice of strains and interpretation of results were criticized by SINGH and HALE (1990). Their most significant points were that (1) there is partial reproductive isolation between Apple Hill and Bogotá strains and (2) Bogotá females show a strong mating preference for Apple Hill males (see also SINGH 1983). SINGH and HALE (1990) suggested that these two factors may be sufficient to explain the observed changes in haplotype frequencies. MACRAE and ANDERSON (1988) stated that they placed previously mated females along with males of the two strains. However, Drosophila females mate more than once, and the sperm of the last male takes precedence over that of previous male(s) (SINGH 1983). Therefore, the use of mated females was equivalent to introducing virgin females and does not overcome the problem of assortative mating, a factor likely to influence haplotype frequencies if males are limiting. SINGH and HALE (1990) concluded that "Sporadic selection in mtDNA is a real possibility with highly diverged mtDNA variants and has important consequence for introduction of mtDNA among isolated populations and across species boundaries. However, unless the interacting genetic systems can be identified, use of the sporadic selection argument to explain variable results would be unwise as there is a danger of confusing real results due to cytonuclear interaction and spurious results due to some uncontrolled aspects of the biological system . . . *It is important that any attempt to understand the role of selection on mtDNA variants should first begin with simpler conspecific variants rather than with interspecific variants*" (p. 997) (emphasis ours). MACRAE and ANDERSON (1990) responded by suggesting that neither mating preferences nor partial reproductive incompatibility were sufficient to explain their results.

FOS *et al.* (1990), in a study on *Drosophila subobscura*, also claimed that the two mtDNA haplotypes they tested were not selectively neutral. They concluded

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that the frequency changes in their cages are "almost exclusively controlled by selection" (p. 4200). They, however, failed to undertake reciprocal crosses to discount the possibility of assortative mating and mtDNA differences "hitchhiking" on nuclear differences in a situation analogous to linkage disequilibrium. The linkage effect decays rapidly so that if there is a strong selection on nuclear DNA, mtDNA frequencies will reflect this selection differential for a few initial generations, after which they may fluctuate randomly or achieve an apparent equilibrium [see ASMUSSEN, ARNOLD and AVISE (1987) for details]. A pattern of an initial rapid increase in the frequency of one haplotype followed by an apparent equilibrium is common to both MACRAE and ANDERSON's (1988) study and FOS *et al.*'s (1990) study. Although it is not known if there were nuclear differences between the *D. subobscura* strains used by FOS *et al.*, it is known that there are nuclear differences between Apple Hill and Bogotá strains of *D. pseudoobscura* (PRAKASH 1972; SINGH and HALE 1990).

A third study to detect selection on mtDNA haplotypes was carried out by NIGRO and PROUT (1990) on two strains of *Drosophila simulans*. Based on cage and simulation studies, NIGRO and PROUT (1990) concluded that changes in haplotype frequencies in their experiments can adequately be accounted for by unidirectional, partial incompatibility without a need to invoke selection on mtDNA. NIGRO and PROUT (1990), like SINGH and HALE (1990), stressed that any studies that seek to test neutrality of mtDNA must be done without the complications of partial reproductive incompatibility.

In this study we report on frequencies of mtDNA haplotypes in laboratory cage populations of the mosquito, *Aedes albopictus* (Skuse) (Diptera: Culicidae). Our objectives were (1) to determine if we could detect selection on mtDNA in *A. albopictus* as has been reported for *Drosophila* spp. and (2) determine the effect of unidirectional cytoplasmic incompatibility on mtDNA haplotype frequencies. We initiated the studies with a pair of fully reproductively compatible lines that differ in mtDNA haplotype and another pair of unidirectionally incompatible lines that also differ in mtDNA haplotype. We hypothesized that (i) if the haplotypes are selectively neutral, frequencies in cages with bidirectionally compatible lines may fluctuate randomly but no one haplotype will consistently increase in frequency and (ii) barring strict assortative mating, the haplotype carried by females that produce inviable eggs upon mating with males of the other line should decrease over time. We had earlier reported on mtDNA variation in *A. albopictus* (KAMBHAMPATI and RAI 1991a) and *Aedes* species (KAMBHAMPATI and RAI 1991b). Incompatibility in *A. albopictus* is modulated by *Wolbachia* sp. (KAMBHAMPATI, RAI and BURGUN 1992).

MATERIALS AND METHODS

Aedes albopictus used in this study were collected in the field either as adults or as eggs several years ago and have been maintained in the laboratory since. The collection and rearing details can be found in KAMBHAMPATI and RAI (1991a).

Two strains were used: Mauritius and Indianapolis (Indiana). KAMBHAMPATI and RAI (1991a) reported that there was restriction fragment length polymorphism in mtDNA of Mauritius population when assayed with the restriction enzyme *Hae*III. Some individuals yielded fragments of 7.6, 6.0, 2.0, 1.2 and 1.1 kbp (mtDNA1) and others fragments of 7.6, 4.3, 2.0, 1.7, 1.2 and 1.1 kbp (mtDNA2). mtDNA1 was also detected in individuals of 16 other populations from around the world; however, mtDNA2 was present only in individuals from Mauritius.

Before cage experiments were initiated, approximately 800 eggs from the Mauritius laboratory colony were hatched and the larvae were reared to adulthood. Adults were allowed to mate for 3 days, after which the females were given a bloodmeal and transferred to individual, numbered cages. The females were allowed to oviposit for 7 days. After oviposition, the females were stored at −70° and the eggs at 10° for future use. DNA from each of these parental females was extracted, digested with *Hae*III and their haplotype determined (see below for methods). Eggs from all females carrying the same haplotype were combined to establish a pure line with regard to mtDNA. After the establishment of these pure lines, two more generations were raised to increase the colony size.

Experiments were done with discrete generations in 20-liter buckets in an insectary at 27 ± 1°, 80 ± 10% relative humidity and a 14-hr photoperiod. Two sets of three cages each were established: the first set of three cages consisted of equal numbers of Mauritius individuals with mtDNA1 and Mauritius individuals with mtDNA2. The second set of three cages consisted of equal number of Indianapolis individuals with mtDNA1 and Mauritius individuals with mtDNA2. We have determined that when Mauritius females mate with Indianapolis males, the resulting eggs do not hatch (KAMBHAMPATI, RAI and BURGUN 1992). The reciprocal cross is fully compatible.

In testing for neutrality of organelle DNA, reciprocal crosses must be performed prior to the initiation of cage populations to avoid hitchhiking of mtDNA on nuclear DNA and to avoid the possibility of assortative mating (see above). Although we did not perform reciprocal crosses expressly for this study, the mosquitoes carrying the two haplotypes in the bidirectionally compatible cages have been raised as a single colony for over a hundred generations. Therefore, it is virtually certain that the nuclear and the mitochondrial backgrounds have been randomized and no nuclear differences exist between the two lines.

To determine if there was mate preference among Mauritius individuals that carry different haplotypes and among Mauritius individuals with mtDNA2 and Indianapolis individuals, mate preference tests were undertaken as follows: 10 mtDNA1 Mauritius females with 20 Mauritius males (10 mtDNA1 males and 10 mtDNA2 males); 10 mtDNA2 Mauritius females with 20 Mauritius males (10 mtDNA1 males and 10 mtDNA2 males); 10 mtDNA2 Mauritius females with 20 males (10 Indianapolis males and 10 mtDNA2 Mauritius males); and 10 Indianapolis females with 20 males

(10 Indianapolis males and 10 mtDNA2 Mauritius males). For each test, three replicates were undertaken. In each of the four tests, the female mosquitoes were first placed in a cup with a meshed lid into which the appropriate males were introduced. The males of one kind were marked with a pink fluorescent dye and the males of the second kind with a green fluorescent dye. The mosquitoes were observed for 10 min or until all the females have initiated mating, whichever was sooner. Immediately after the initiation of a mating, the mating pair was aspirated and stored for later identification. The males were identified by determining the color of the dye under UV light. The observed values were compared against expected random mating using a chi-square test. For all tests and replicates, the statistical analysis indicated that the matings were random.

In the first set of experiments, the parental generation for each of the three replicate cages consisted of 150 males and 150 females each of Mauritius individuals with mtDNA1 and mtDNA2. These were introduced into cages at the pupal stage (earliest stage at which the sexes can be separated). In the second set of experiments with unidirectional incompatibility, the parental generation for each of the three replicate cages consisted of 150 males and 150 females of Indianapolis with mtDNA1 and 150 males and 150 females of Mauritius with mtDNA2. Three days post emergence, the females were bloodfed and allowed to oviposit for 7 days. At the end of oviposition, a portion of eggs was hatched and reared to pupal stage. At this stage, 300 males and 300 females were counted and reintroduced into the respective cages for eclosion and the cycle was repeated. The experiments were carried out for a total of 10 generations. At the end of each generation, all adults in the cage were frozen at −70° for analysis of mtDNA.

DNA was isolated from each individual adult female mosquito according to COEN, STRACHAN and DOVER (1982). The sample sizes per generation and per cage ranged from 36 to 88 individuals with a mean of (± SD) 57 ± 13 for cages with bidirectionally compatible lines and 50 ± 6 for cages with unidirectionally incompatible lines. Mosquitoes were sampled at alternate generations, *i.e.*, F_2, F_4, F_6, F_8 and F_{10}. After extraction, the DNA was digested with the restriction enzyme *Hae*III, separated on 1% agarose gels in TBE buffer (SAMBROOK, FRITSCH and MANIATIS 1989) and Southern blotted (SOUTHERN 1975) onto nylon membranes. The DNA on the membranes was probed with radiolabeled mtDNA of *Anopheles quadrimaculatus*, which had previously been cloned (COCKBURN, MITCHELL and SEAWRIGHT 1990). After washing and drying (see KAMBHAMPATI and RAI 1991a for details), the membranes were exposed to X-ray film for 1–3 days. The number of individuals carrying mtDNA1 or mtDNA2 haplotypes at each generation was determined.

The observed frequency of haplotypes was tested for departure from initial frequency of 50% by a chi-square test. For each generation and cage, the null hypothesis that observed frequencies can adequately be explained by genetic drift alone was tested following the procedures of FISHER and FORD (1947). FISHER and FORD (1947) have shown that the effective population size (N_e) at each generation and the sample taken from this effective population, can be used to derive an expected covariance matrix of gene frequencies observed in the various generations. By means of a chi-square, the matrix can be used to test the null hypothesis that observed fluctuations can be expected under an assumption of random genetic drift alone. We considered N_e equivalent to the number of females used to start each generation (*i.e.*, 300), an assumption shown to be a good approximation for a maternally inherited genome (BIRKY, MARUYAMA and FUERST 1983).

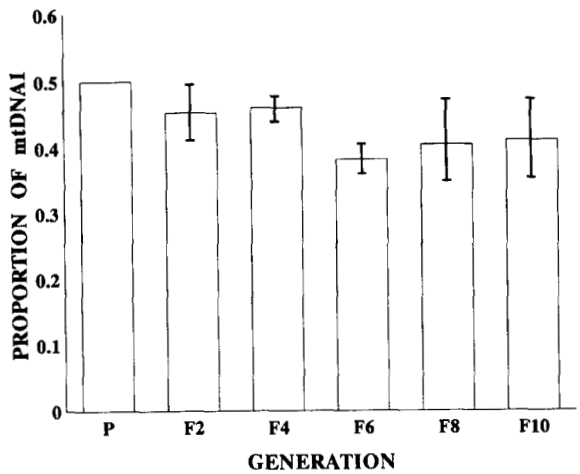

FIGURE 1.—The mean proportion of mtDNA 1 from three replicate cages with bidirectionally compatible strains. The error bars represent ± 1 SEM.

RESULTS

The results from the two sets of cages are shown in Figures 1 and 2. In cages with bidirectionally compatible lines (Figure 1), frequency of the haplotypes fluctuated from one generation to the next. However, there was no consistent pattern to these fluctuations. The frequency of the haplotypes departed from the initial 50% occasionally. Of the 15 chi-square tests, 5 (33%) were found to be significant ($P < 0.05$). These were: cages 1 and 3, generation F_6; cages 2 and 3, generation F_8; and cage 2, generation F_{10}.

However, FISHER and FORD's (1947) test indicated that the observed magnitude of fluctuations in haplotype frequencies in cages with bidirectionally compatible lines can be explained under an assumption of random genetic drift alone. The chi-square test was nonsignificant ($P > 0.05$) both when the proportions from each cage were considered individually (chi-square values: cage 1, 4.40; cage 2, 5.20; cage 3, 5.49; d.f.= 4) and when the proportions for all three cages were considered together (chi-square 2.91; d.f.= 4).

In cages with unidirectionally incompatible strains, haplotype frequency changes were dramatic (Figure 2). The frequency of mtDNA2 (present in Mauritius females) dropped to zero in the F_2 generation. Based on this observation, we assayed, *a posteriori*, 36 F_1 individuals to determine when the frequency changes occurred. We detected individuals with mtDNA2 in low frequency (4/36) in cage 2 and were unable detect any in cages 1 and 3. This indicated that the shift in frequencies began to occur in the parental generation and mtDNA2 was completely replaced in the second generation.

DISCUSSION

Our results indicated that (i) in cages with bidirectionally compatible lines, frequencies of the mtDNA

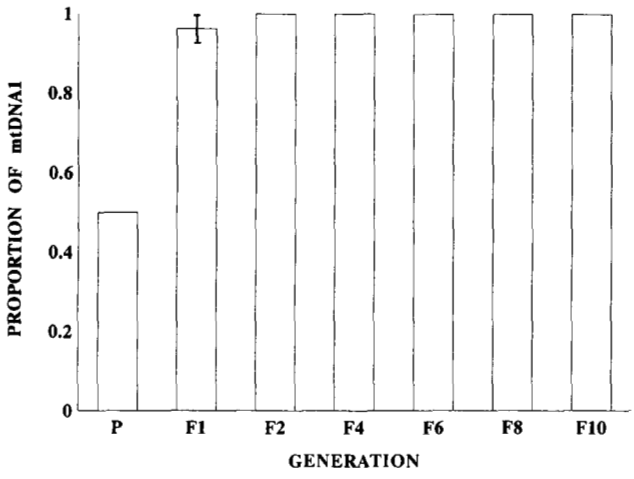

FIGURE 2.—The mean proportion of mtDNA1 from three replicate cages with unidirectionally incompatible strains. The error bar represents ± 1 SEM. After the F_1 generation, mtDNA2 could not be detected in any of the 3 cages.

haplotypes fluctuated randomly with no indication of one haplotype replacing the other and (ii) in cages with unidirectionally incompatible lines, the frequency of mtDNA2 decreased dramatically in F_1 generation and was replaced completely in the F_2 generation. The results in both sets of cages are consistent with null hypotheses stated earlier.

Unlike MACRAE and ANDERSON (1988) and FOS *et al.* (1990), we did not find evidence for selection on mtDNA haplotypes in *A. albopictus*. The 5/15 significant deviations from the initial frequency of 50% were the result of random genetic drift as indicated by the covariance matrix analysis. The chi-square values derived from the covariance matrix were non-significant ($P > 0.05$) both when the frequencies in each individual cage were considered and when the data were combined for all three cages.

Considering that both MACRAE and ANDERSON (1988) and FOS *et al.* (1990) did not observe the supposed evidence for selection on haplotypes in all of their replicate cages, a more parsimonious explanation for their observations is stochastic lineage survivorship (AVISE, NEIGEL and ARNOLD 1984), mate preference (SINGH and HALE 1990), partial unidirectional incompatibility (NIGRO and PROUT 1990) and/or their failure to adequately discount the possibility of mtDNA haplotypes hitchhiking on nuclear differences. MACRAE and ANDERSON (1988) estimated that only 1 in 10 larvae reached reproductive age, providing ample opportunity for random mortality that may be reflected in the frequency of haplotypes. Regardless of the probable causes, it is clear that there were a number of extraneous factors in both MACRAE and ANDERSON's (1988) study and FOS *et al.*'s (1990) study that may have influenced the outcome. Therefore, it is too early to state conclusively that the observed haplotype frequency changes are a direct result of selection. Until their results can be replicated in studies without the complications mentioned above, or precise fitness values can be ascribed to each haplotype, it is prudent to assume that minor differences among haplotypes are selectively neutral.

The selective neutrality of haplotypes, however, does not necessarily assure the survival all haplotypes over evolutionary time. Random processes, natural selection on attributes unrelated to mtDNA or factors unrelated to the genetics of the organism could lead to the predominance of one haplotype in a species (AVISE, NEIGEL and ARNOLD 1984). An indication of extraneous factors influencing haplotype frequency was found in cages with unidirectionally incompatible strains. The changes in haplotype frequencies were rapid and in the direction expected, with mtDNA2 being replaced completely in two generations. While we had hypothesized that the frequency of the mtDNA2 would decline over time, we were surprised how quickly it was driven to extinction. As mentioned above, the mate choice experiments indicated that the matings were random. However, the results from the cages suggest that a substantive proportion of Mauritius females must have mated with Indianapolis males very early in the experiments. In any case, mtDNA2 was driven to extinction not due to selection differentials among haplotypes, but as a direct consequence of cytoplasmic incompatibility modulated by the endosymbiont, *Wolbachia* sp.

In a simulation study, ROUSSET, RAYMOND and KJELLBERG (1991) have shown that in a large, panmictic population that is polymorphic for n cytotypes, a stable polymorphism cannot exist between incompatible cytotypes. One cytotype will inevitably go extinct and the process of cytotype elimination will continue with $n - 1$ cytotypes until all coexisting cytotypes are compatible. Only a strict assortative mating among compatible cytotypes will allow for the existence for cytotype polymorphism. The results from cages with unidirectionally incompatible lines support the conclusion that incompatible cytotype polymorphism is not stable even over very short periods of time. Those females that can produce viable eggs with more than one kind of male (in this case Indianapolis females) clearly have a selective advantage and can be expected to spread through a population rapidly. Recently, TURELLI and HOFFMANN (1991) presented evidence that Wolbachia infection is spreading rapidly in field populations of *D. simulans* in California.

Endosymbiont-modulated incompatibility has been reported to-date from a wide range of insect genera including *Aedes*, *Culex*, *Nasonia*, *Drosophila*, *Tribolium*, *Hypera* and *Laodelphax*. In addition, a number of other insect genera are known to be infected with the microorganism, although cytoplasmic incompatibility has not yet been reported for them (O'NEILL *et al.*

1992). This suggests that observations that one haplotype is predominant throughout the range of a species must take into consideration the role of Wolbachia or similar microorganisms in influencing the pattern of mtDNA variation. This point was also stressed by HALE and HOFFMANN (1990), who in a study of *D. simulans*, found a strong association between incompatibility type and mtDNA variation. The influence of cytoplasmic incompatibility on mtDNA frequencies may be reflected in at least two ways. In organisms that have been infected for an evolutionarily long period of time, one haplotype is likely to predominate throughout the range of a species, given significant gene flow. For example, KAMBHAMPATI and RAI (1991a) have shown that one haplotype predominates throughout the range of *A. albopictus*. The only populations that contained different haplotypes were those on isolated islands. This raises the possibility that cytoplasmic incompatibility may have played a role influencing the frequency of mtDNA haplotypes in *A. albopictus*. In cases of evolutionarily recent infections and/or no significant gene flow, a sharp population subdivision with regard to haplotypes can be expected as was observed in *D. simulans* by HALE and HOFFMANN (1990). Even in cases of recent infections, with adequate amounts of gene flow, it is to be expected that the mtDNA of females that are compatible with 2 or more types of males will eventually predominate throughout the range of a species. An example is the study by TURELLI and HOFFMANN (1991) who documented the spread of Wolbachia-infected *D. simulans* in California.

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