

# Further observation of paternal transmission of *Drosophila* mitochondrial DNA by PCR selective amplification method

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(Received 19 June 1991 and in revised form 25 November 1991)

## Summary

By designing 3' ends of primers in PCR (polymerase chain reaction), a specific DNA fragment was selectively amplified in the presence of a  $10^3$ -fold excess of highly homologous (sequence difference ca. 2%) opponent DNA. This technique was applied in detecting paternal leakage of mitochondrial DNA (mtDNA) in intraspecific crosses of *Drosophila simulans* and interspecific crosses of *Drosophila simulans* and *Drosophila mauritiana*. The mtDNA types of their progeny were analysed by selective amplification of the paternal mtDNA fragment possessing a polymorphic restriction site and detecting its cleaved fragments. Paternal mtDNA was detected in the progeny of 14 out of 16 crosses. The present result indicates small but frequent inheritance of sperm mtDNA in *Drosophila*, which is supportive to our previous finding.

## 1. Introduction

Inheritance of mtDNA is complicated by the multiplicity of mtDNA within a mitochondrion and a cell. The details of mtDNA segregation between mitochondria and between cells, and selection operating on mtDNA within and between generations are still unclear. Mitochondrial DNA inheritance has been believed to be strictly maternal, although some exceptions are found in plants and animals (Mirfakhrai *et al.* 1990; Neale *et al.* 1990; Kondo *et al.* 1990; Hoeh *et al.* 1991; Gyllensten *et al.* 1991). Theoretical population genetics studies have suggested that transmission of even a small amount of paternal mtDNA can provide a sufficient extent of gene flow between otherwise isolated female lineages, which may influence the evolutionary dynamics and history of maternal lineages considerably (Takahata & Maruyama 1981; Chapman *et al.* 1982; Birky 1983).

One of the first genetic evidences of paternal mtDNA transmission was found in *Drosophila*. By means of Southern hybridization, sperm mtDNA was detected in the progeny of backcrossed lines using different mtDNA types (Kondo *et al.* 1990). The probe constructed for this experiment required longer exposure of the autoradiograph to detect paternal mtDNA in an amount at least 0.03% of a mixture with maternal mtDNA. A PCR method may provide

an easier and more efficient method with which to further analyze the paternal inheritance of mtDNA, owing to its capacity to amplify specific segments from crude and minute quantities of DNA (Saiki *et al.* 1985; Gyllensten *et al.* 1990), and its sensitivity which allows one to dispense with radioactive probes (Kaneko *et al.* 1989). Recent reports point out that perfectly matched primers are favoured over mismatched primers in amplification (Gibbs *et al.* 1989), and that base pairing at the 3' end of a primer is particularly crucial (Sommer & Tautz 1989). It has been demonstrated that one can discriminate two alleles that differ by a single nucleotide by using primers that have allele-specific nucleotides in their 3' ends (Wu *et al.* 1989).

In the present study, we designed the 3' end of primers to distinguish two mtDNA types in *Drosophila*. The ability to amplify a specific segment from the perfectly matched mtDNA in the presence of abundant opponent DNA was examined. This was further applied in the study of mtDNA inheritance and the detection of paternally inherited mtDNA in *Drosophila*.

## 2. Materials and methods

Isofemale lines of *Drosophila simulans* (SI303, SI307 and SI265) and *Drosophila mauritiana* (g20) were established from individual inseminated females which were collected in 1979 from Réunion (St Denis) and Mauritius (Port Louis), respectively. SI303 and SI307

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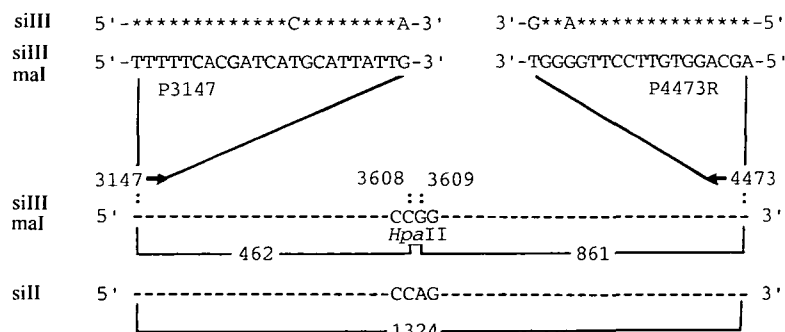


Fig. 1. Design of oligonucleotide primers and expected size of amplified fragment after *HpaII* digestion. Sequence for the primers were taken from mal(siIII) mtDNA and the region amplified is numbered (3147–4473) as in the sequence of *Drosophila yakuba* mtDNA (Clary & Wolstenholme 1985). Total length is 1324 bp in siII and 1323 bp in siIII(mal) due to deletions in siII and siIII(mal) mtDNA (Kaneko & Chigusa, unpublished data). Homologous sequence of siII mtDNA is aligned above each primer sequence. Asterisks indicate the sequence identity with mal(siIII) mtDNA. The 3' ends of both primers differ from the homologous sequences of siII mtDNA enabling selective amplification of mal(siIII) mtDNA.

represent siII mtDNA type, SI265 and g20 represent siIII and mal mtDNA types. The two mtDNA types of *Drosophila simulans*, siII and siIII, differ in many restriction sites (Solignac *et al.* 1986) and the sequence difference between the two mtDNA fragments (2.5 kb long) is about 2% (Satta & Takahata 1990). However, the siIII mtDNA type shows extremely high homology with mal mtDNA type of *Drosophila mauritiana* (99.6%) (Satta & Takahata 1990; Kaneko & Chigusa, unpublished data) and is not distinguishable by restriction patterns (Solignac *et al.* 1986).

In designing efficient primers with which to discriminate mal(siIII) from siII, we considered the following conditions in addition to the general rules (Innis *et al.* 1990). First, both primer sequences must be perfectly homologous to the mal(siIII) mtDNA but differ from the siII mtDNA at their 3' ends. Second, the amplified segment must contain a polymorphic restriction enzyme cleavage site for easy confirmation of the sequence. Oligonucleotide primers P3147:

5'-TTTTTCACGATCATGCATTATTG-3'

and P4473R:

5'-AGCAGGTGTTCTTGGGGT-3'

were synthesized with an Applied Biosystems 381A DNA synthesizer. These primers satisfy these conditions for selective amplification of a 1281 bp sequence (1323 bp including the primers) of mal(siIII) mtDNA (Kaneko & Chigusa, unpublished data).

To obtain efficient annealing of these primers to the target sequence, mtDNA was extracted through SDS-phenol treatment of mitochondria isolated from homogenates of adult flies and used as templates for the PCR reaction. The reaction was performed, using a DNA thermal cycler (Perkin Elmer/Cetus), in 100  $\mu$ l of a solution containing 50 mM-KCl, 10 mM-Tris pH 8.3, 1.5 mM-MgCl<sub>2</sub>, 0.001% of gelatin, 200  $\mu$ M each of dNTP, 0.5  $\mu$ M each of primers, approximately 150 ng

of template mtDNA and 2.5 units of *Taq* polymerase, covered with 50  $\mu$ l of mineral oil. The following condition was used: 20 s at 94 °C, 20 s at 55 °C and 30 s at 72 °C for 30 cycles and 7 min at 72 °C afterwards; 1/10 volume (10  $\mu$ l) of each PCR product was applied on a gel. The mtDNA type was confirmed by digestion of the amplified fragment with *HpaII*: mal(siIII) mtDNA is cleaved into 462 and 861 bp whereas 1324 bp of siII mtDNA remains undigested (Fig. 1).

### 3. Results and discussion

Control experiments were performed using mtDNA from SI303 and g20 as representatives of the maternal siII and the paternal mal(siIII) mtDNA, respectively. The efficiency of amplification was tested first in a series of PCR reactions by decreasing the amount of template mal mtDNA from 6 ng to 0.025 pg. Under the conditions used here, a gradual decrease in the amount of product was observed with amounts of template below 0.3 ng. 25 pg of the template (mal mtDNA) yielded sufficient product for detection after 30 cycles. Approximately 2.5 pg of mal mtDNA was enough, when 5  $\mu$ l of PCR product was used as template for additional 30 cycles (or a total of 60 cycles) (Fig. 2, lanes 2, 3).

The capacity for selective amplification was examined with siII mtDNA in a similar way. There was no sign of the amplified fragment in the whole PCR product from *ca.* 150 ng of siII mtDNA template after a total of 60 cycles (Fig. 2, lane 7). Therefore, mismatches in the 3' ends of primers prevent amplification of the siII mtDNA under these conditions. However, siII mtDNA could be amplified under less stringent conditions, such as lowering the annealing temperature.

Finally, to test the effect of competition between the two templates, a series of reactions were made containing different amounts of mal and an excess

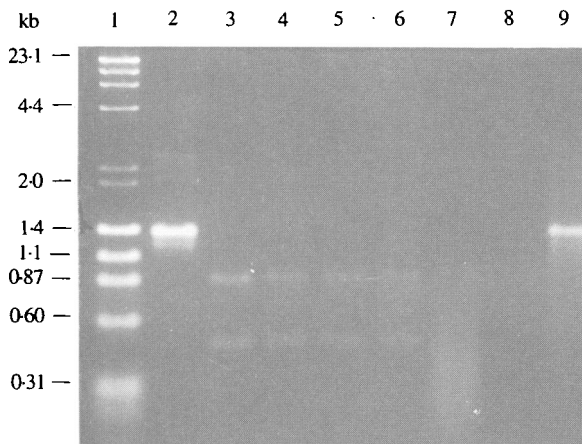


Fig. 2. Restriction analysis of the amplified products. After amplification, mineral oil was completely removed with chloroform, DNA was then precipitated with ethanol. The amplified fragment was purified by gel electrophoresis and digested with *Hpa* II. It was then applied to 1.5% agarose gel and visualized with ethidium bromide staining; 10  $\mu$ l of the product was applied on a gel when undigested. The content for each lane is as follows: 1, DR1gest III molecular marker (Pharmacia); 2, product of g20 undigested; 3, product of g20 digested with *Hpa* II; 4, product of sm1CII digested with *Hpa* II; 5, product of sm1CI digested with *Hpa* II; 6, product of sm2CI digested with *Hpa* II; 7, concentrated whole product of SI303 (template = 150 ng) digested with *Hpa* II (60 cycles); 8, product of SI303 (template = 25 ng) + g20 (template = 2.5 pg) undigested (60 cycles); 9, product of SI303 (template = 25 ng) + g20 (template = 25 pg) undigested.

(approximately 25 ng) of siII mtDNA. Approximately 25 pg of maI mtDNA yielded sufficient maI mtDNA product after 30 cycles, but, a reaction starting with about 10-fold less (*ca.* 2.5 pg) maI mtDNA failed to yield a detectable amount of product even after 60 cycles of amplification (Fig. 2, lanes 8, 9). This shows that maI mtDNA can be amplified in presence of at least  $10^3$  excess of siII mtDNA.

These primers were applied in the detection of paternally inherited mtDNA. In a previous study, paternally derived mtDNA was detected after ten consecutive backcrosses (Kondo *et al.* 1990). There, backcrossing was performed taking into account two possible effects. First, if a small amount of paternal mtDNA is inherited at each generation, backcrossing may serve to accumulate it to the point of detectability. Second, the nuclear genome of a backcross line is replaced by that of the donor male which may be favourable to the replication of paternally derived mtDNA. On the other hand, as selection operating on mtDNAs during backcross generations is not known, this can be a nuisance when assessing the amount of paternal leakage. In this study,  $F_2$  progeny obtained after one to two matings with the paternal strain was examined.

There are four possible combinations between siII females and maI(siIII) males. Two intraspecific crosses [SS1 – SI303 (female)  $\times$  SI265 (male); ss2 – SI307 (female)  $\times$  SI265 (male)] and two interspecific crosses

[sm1 – SI303 (female)  $\times$  g20 (male); sm2 – SI307 (female)  $\times$  g20 (male)]. For each combination, two crosses, Cross I (CI) and Cross II (CII), were made. CI is a backcross where a  $F_1$  virgin female was crossed to males from the paternal strain. Paternal mtDNA detected in this cross would have been derived from that incorporated into an egg at the first or the second mating and have remained in the adult  $F_2$  progeny. If it were derived solely from the second mating, this would not indicate actual inheritance of paternal mtDNA. The inheritance of the paternal mtDNA incorporated into germ cells of  $F_1$  progeny is examined through CII. For ss1 and ss2, a  $F_1$  virgin female was crossed with its siblings ( $F_1$  males). For sm1 and sm2, males from the maternal strain were used instead, since the interspecific hybrid male is sterile. All crosses were performed between a single female and 2–3 males at 25 °C so as to follow each maternal lineage.

Mitochondrial DNA of  $F_2$  progeny from a total of 16 experimental lines were examined, two each from the eight crosses (ss1CI, ss2CI, sm1CI, sm2CI, ss1CII, ss2CII, sm1CII, sm2CII). Mitochondrial DNA from approximately 50 adult flies (*ca.* 150 ng) was used for the template. After 60 cycles of amplification, a 1.3 kb fragment was detected from 14 lines, the two exceptions were from crosses ss2CI and sm2CI (results not shown). The amplified DNAs from four lines (a line each from cross sm1CI, sm2CI, ss2CII, sm1CII) were subjected to *Hpa* II digestion and separated on agarose gel (Fig. 2, lanes 4–6). The fragment was cleaved completely indicating that paternal mtDNA fragments were amplified selectively in all four lines. The absence of amplified band from control siII carried out at the same time eliminates the possibility of contamination (Fig. 2, lane 7).

This experiment uses a different method to that of Kondo *et al.* (1990) but again detects paternal leakage of mtDNA in 7 out of 8 lines from both intra- and interspecific crosses. The present result suggests several features of mtDNA inheritance in *Drosophila*. Firstly, paternally derived mtDNA is inherited to the next generation. Although formation of mitochondria in sperm differs from that in the egg, this may suggest the capacity of replication of sperm mtDNA after transmission. The presence of paternal mtDNA in smCII lines indicates that paternal mtDNA is incorporated into germ cells at the first fertilization. Secondly, paternal leakage not only occurs in interspecific but also in intraspecific crosses in *Drosophila*. Paternal inheritance have recently been indicated in mice by use of PCR (Gyllensten *et al.* 1991). After a minimum of 8 backcross generations, the heteroplasmic state persisted for 14 generations. However, this was in interspecific crosses in each case. Our observation is the first to demonstrate paternal leakage in intraspecific crosses in animals. Since in natural population, paternal leakage of intraspecific crosses can be expected more frequently than that of interspecific crosses, this may have more evolutionary



significance (Matsuura *et al.* 1991*a*). Thirdly, the paternal leakage revealed here may open interesting investigations on the fate of the male mtDNA. Elimination of foreign paternal mtDNA by maternal nuclear DNA has not been seen. Although the proportion of maternal nuclear DNA in F<sub>2</sub> progeny is 25% in CI and 50–75% in CII, paternal DNA was detected from all of the CII lines. More extensive examination is needed to determine the effect of nuclear background on mtDNA inheritance (Matsuura *et al.* 1991*b*). Finally, the level of paternal leakage is very low, but the paternal input is occurring rather frequently. Given that 25 pg of paternal mtDNA is detectable in a sample, the resolution of the present experiment is estimated to be 0.017% = 25/150000. As a result, paternal mtDNA was detected from 87.5% = 14/16 of the experimental lines. While in our previous study, paternal mtDNA was detected from 1.69% = 4/237 of the experimental lines at the resolution of 0.035% but none at 0.075%. This increase in the rate of detection suggests a frequent inheritance of paternal mtDNA and its low proportion in progeny, which was not detectable using other methods.

This experimental system provides an easy, efficient and direct way to examine the inheritance of mtDNA in *Drosophila*. Studies on mtDNA transmission using PCR method should give further insight to the understanding of the mode of mtDNA inheritance, and the evolutionary consequence of this gene flow.

We would like to thank Dr N. Takahata and Dr Y. Satta for helpful discussions. We thank the two anonymous reviewers for suggesting ways to make the manuscript clearer and more accurate. This work is supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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