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Evolution of the mitochondrial ATPase 6 gene in *Drosophila*: unusually high level of polymorphism in *D. melanogaster*

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Summary

We have determined 1990 bp mitochondrial DNA sequence which extends from 3′ end of the cytochrome oxidase subunit I (COI) gene to 5′ end of the COIII gene from two sibling species of *Drosophila*, *D. simulans* and *D. mauritiana*. Analyses of the sequences and part of the NADH dehydrogenase subunit 2 gene and the COI gene together with those from *D. melanogaster* and *D. yakuba* revealed that amino-acid substitution rate of the ATPase 6 gene seems to be higher in some strains of *D. melanogaster* than in the other species. High level of amino-acid polymorphism in this gene was observed in *D. melanogaster*. Synonymous substitution rate is relatively constant in all the genes examined, suggesting that mutation rate is not higher in the ATPase 6 gene of *D. melanogaster*. The amino-acid substitutions found specifically in *D. melanogaster* are at the sites which are not conserved among mammals, yeast and *E. coli*. These sites of the ATPase 6 gene might lose the selective constraint in *D. melanogaster*, and the amino-acid substitutions can be explained by neutral mutations and random genetic drift.

1. Introduction

The rate of amino acid or nucleotide substitution is approximately constant per site per year for various evolutionary lineages, as long as the function of the gene remains the same. In some molecules, however, it is known that the evolutionary rate of certain species is different from other species. One of the causes for this inconstancy appears to be the change of mutation rate which is most likely to be brought about by the 'generation effect' (Wu & Li, 1985; Kimura, 1987).

Another cause of the change in molecular evolutionary rate may be the alteration of the selective constraint in a protein molecule. For instance, the rapid evolution of guineapig insulin is suggested to be due to a change in the selective constraint (King & Jukes, 1969; Jukes, 1979; Blundell *et al.* 1971). From the nucleotide sequence analysis of a mtDNA coding gene, cytochrome *b*, Kocher *et al.* (1989) have found that the amino-acid substitution rate is about five times lower in the lineage leading to fish than in those leading to birds or rodents. But there is no evidence of positive selection for mutation in a particular lineage.

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We compared nucleotide sequences and amino-acid sequences of four protein coding genes (ND2, COI, COII and ATPase 6) in mitochondrial DNA (mtDNA) from *D. melanogaster*, *D. simulans*, *D. mauritiana* and *D. yakuba*. These species belong to the *melanogaster* subgroup of the genus *Drosophila*. Considering various kinds of data, such as geographical evidence, unique DNA sequences, chromosomal binding pattern, allozymes, rDNA organization and mtDNA RFLP, Lachaise *et al.* (1988) estimated that *D. yakuba* diverged from the other three species 6–15 Mya, and *D. melanogaster* diverged from *D. simulans* and *D. mauritiana* about 2·5 Mya. Divergence time of *D. simulans* and *D. mauritiana* was estimated to be 0·4–2·5 Mya from the nucleotide sequences at the alcohol dehydrogenase locus (*Adh*) (Bodmer & Ashburner, 1984; Ashburner *et al.* 1984). However, these estimates are still ambiguous because of the close phylogenetic relationships among the four species.

From the present analysis, we have discovered that the amino-acid substitution rate of ATPase 6 in some strains of *D. melanogaster* seems to be higher than in the other species compared. ATPase 6 is a subunit of H^+-ATPase in mitochondria. This multisubunit enzyme consists of extrinsic membrane complex, F_1 containing the catalytic sites, and membrane embedded part, F_0 containing the proton channel. ATPase 6 is a component of F_0, and the homologues

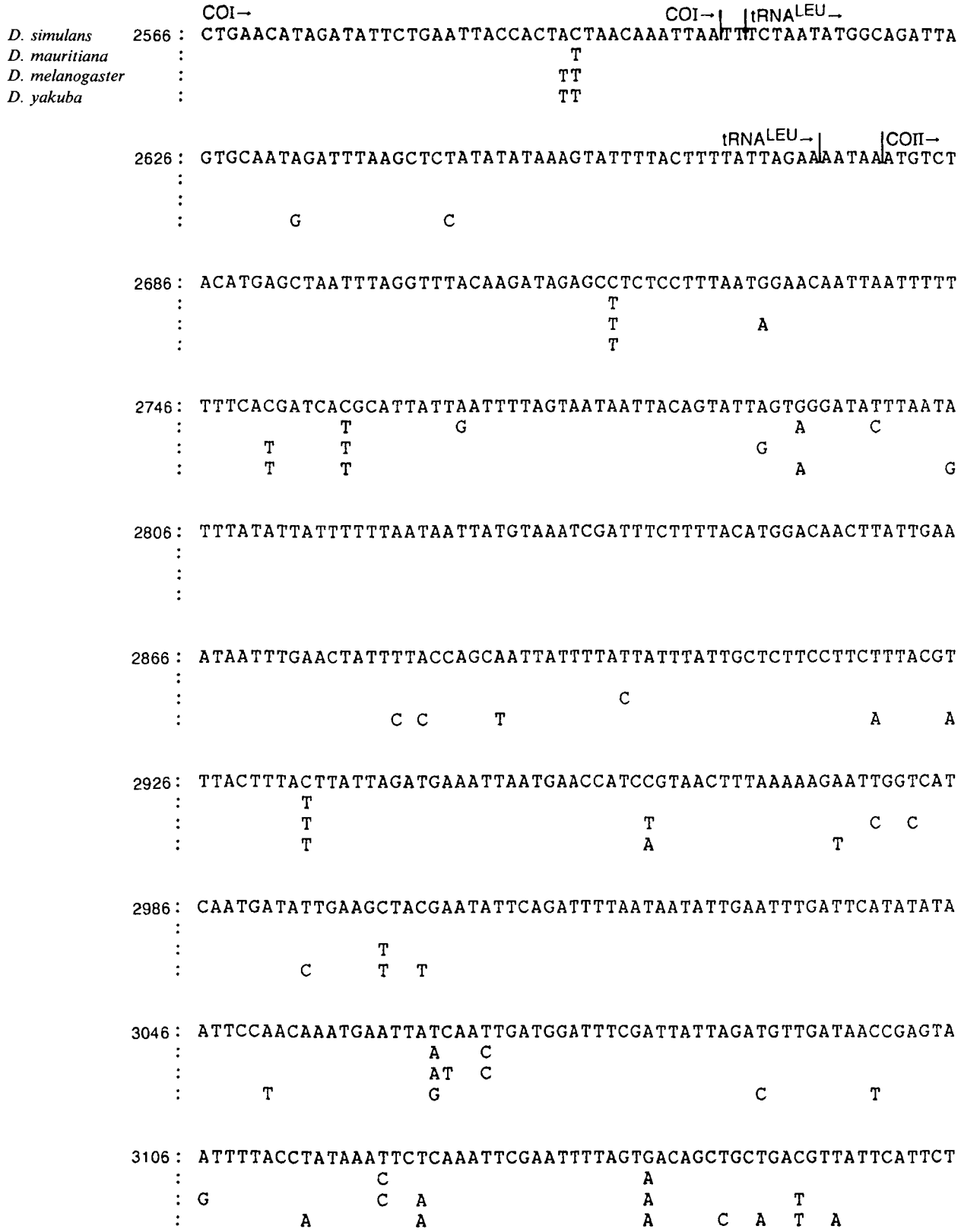

Fig. 1. Sequences of 1990 bp fragments of *Drosophila* mtDNAs. The *D. simulans* sequence is shown on the top line. Differences from this sequence in the *D. mauritiana*, *D. melanogaster* and *D. yakuba* sequence are shown below, and gaps are indicated by —. Vertical lines above the sequences represent 5′ and 3′ ends of the six genes. The direction of each transcript is indicated by an arrow. The positions of the synthetic primers used in the sequencing of the ATPase 6 gene from the two strains of *D. melanogaster*, SP-1 and IR-17, are underlined (solid line, sense strand; broken line, anti-sense strand).

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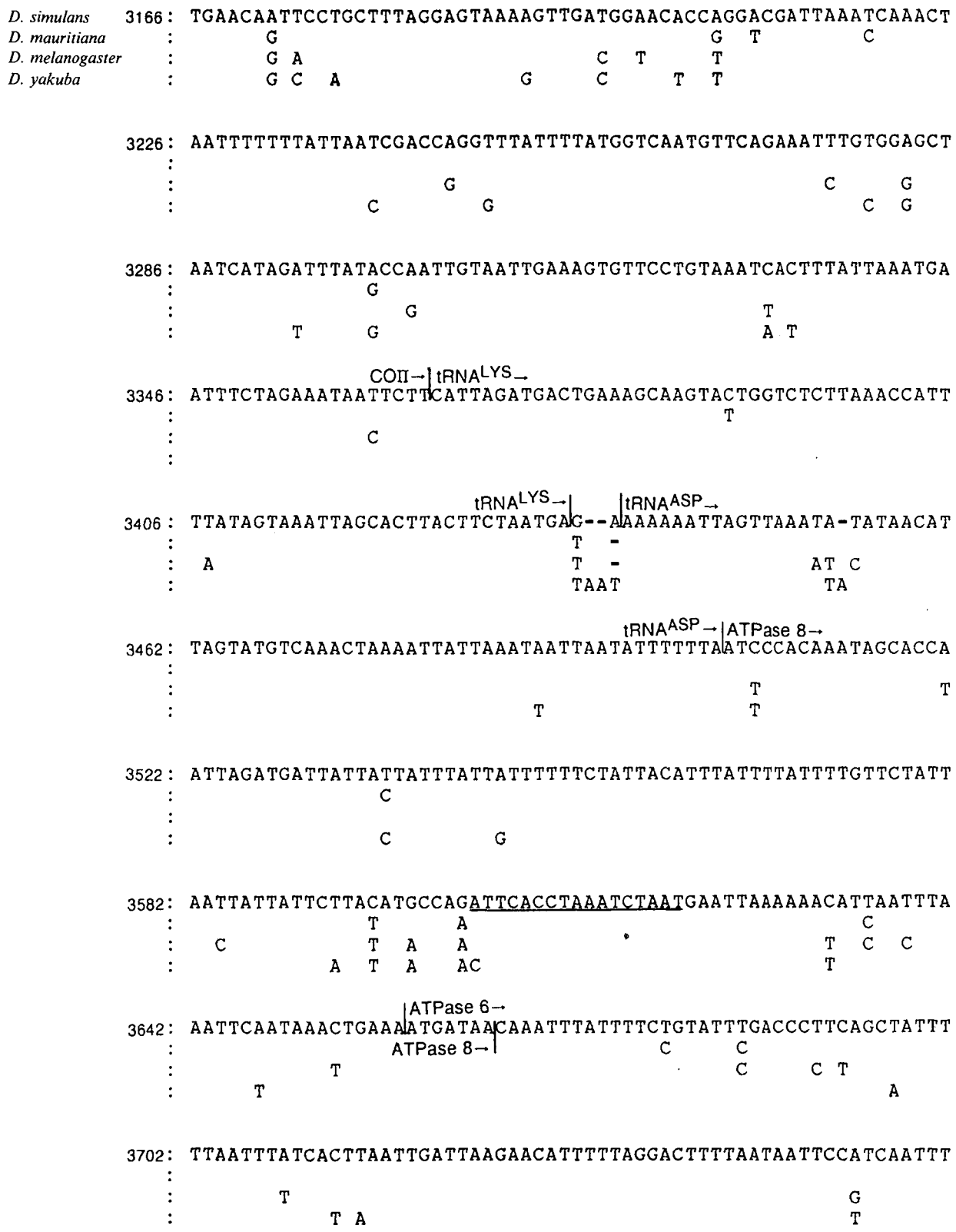

Fig. 1 – *cont.*

are identified not only in mtDNA but also in bacterial DNA and chloroplast DNA (Walker *et al.* 1984; Cozens *et al.* 1986). The transmembrane structure of ATPase 6 protein is predicted to be folded several times in the membrane from the amino-acid sequences of *E. coli* and human counterparts (Cox *et al.* 1986).

The function of ATPase 6 must be well conserved, because it is a component of one of the house-keeping enzymes. We will discuss the change in the evolutionary rate of such a protein in a relatively short time from the viewpoint of molecular evolution, considering the function and structure of the molecule.

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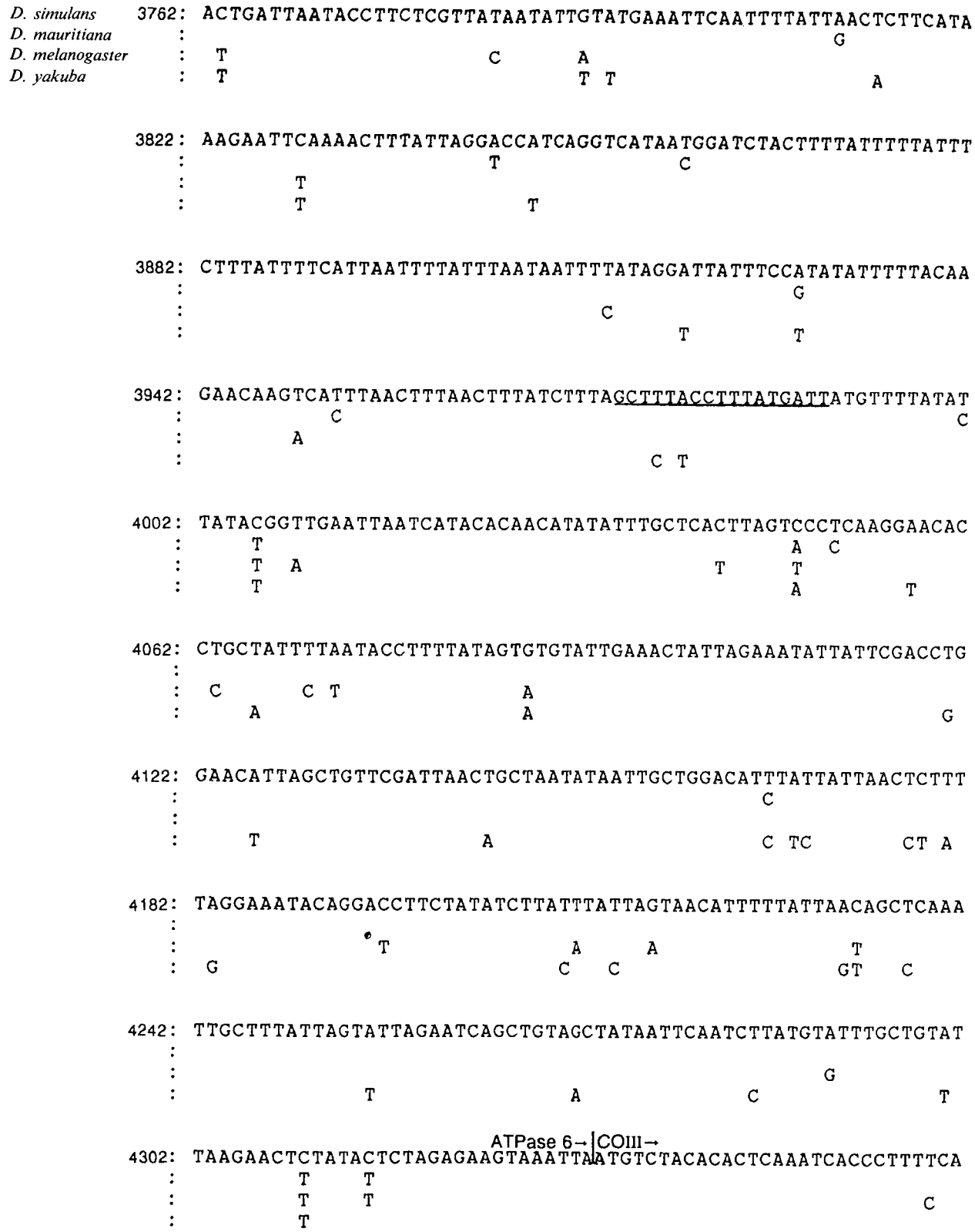

Fig. 1 – *cont.*

2. Materials and methods

(i) *DNA sequences*

The DNA sequences of *D. melanogaster* and *D. yakuba* were from de Bruijn (1983), and Clary & Wolstenholme (1985), respectively. Both of the two mtDNA sequences of *D. melanogaster* from de Bruijn (1983) have Oregon-R background (de Bruijn, 1983). For *D. simulans* and *D. mauritiana*, the DNA sequences of most part of the ND2 and COI genes were from Satta *et al.* (1987) and Satta & Takahata (1990). To examine the COII and ATPase 6 and 8

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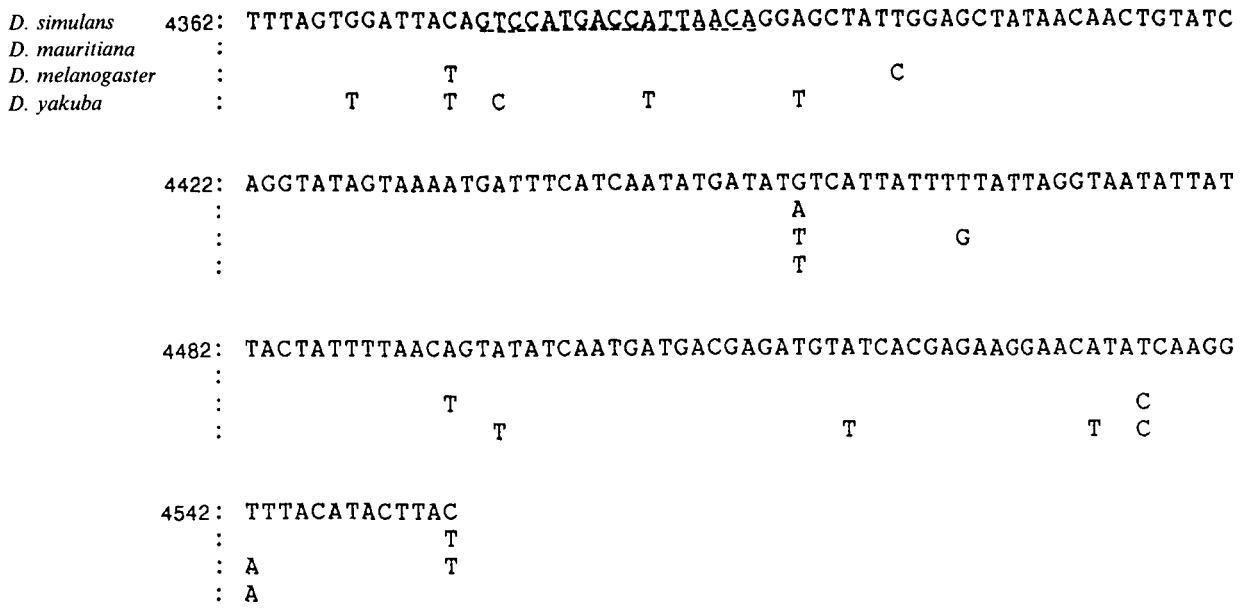

Fig. 1 – *cont.*

genes of *D. simulans* and *D. mauritiana*, we have determined the DNA sequences of 1990 bp segments containing three tRNA genes (tRNA Leu, Lys and Asp), three complete protein genes, COII and ATPase 6 and 8, and parts of the two protein genes, COI and COIII, for the two species. For each of the two species, we have sequenced mtDNA from one isofemale line collected by O. Kitagawa in 1979. The strain of the cosmopolitan species, *D. simulans*, was collected in Nairobi, Kenya. *D. mauritiana* is endemic to Mauritius. The 4·9 kb *Hin*d III fragments from *D. simulans* and *D. mauritiana* mtDNA were cloned in pHY300PLK (Ishiwa & Shibahara, 1985) by Y. Kubota. These clones were amplified, purified (Maniatis *et al.* 1982), and digested with *Pvu* II, *Eco*R I, and *Hin*d III. *Pvu* II–*Pvu* II (580 bp), *Pvu* II–*Eco*R I (679 bp), *Eco*R I–*Pvu* II (441 bp) and *Pvu* II–*Hin*d III (600 bp) fragments from the clones were subcloned into M13mp18 and mp19 (Messing *et al.* 1981). Each fragment was inserted into the vector in both directions except the *Pvu* II–*Hin*d III (600 bp) fragment for which we have sequenced only 288 bp. For each of the *Pvu* II–*Pvu* II (580 bp) and *Pvu* II–*Eco*R I (679 bp) fragments, we made a deletion of about 120 and 200 bp, respectively, to confirm the sequence. The DNA sequences of the resulting ssDNAs were determined several times from both directions by the dideoxy chain termination method using a primer commercially available (Sanger *et al.* 1977).

To examine the polymorphism in the ATPase 6 gene of *D. melanogaster*, we have determined the DNA sequences of the ATPase 6 gene for two isofemale lines of *D. melanogaster*. Both were collected in Japan in 1982, one from Sapporo (SP-1), and the other from Iriomote (IR-17). Mitochondrial DNA extracted from each of the two strains was digested with *Hae* III and *Hin*d III. The 1885 bp fragment was cloned into M13mp10 or mp18 and mp19. The DNA sequences of the resulting ssDNAs were determined by the dideoxy chain termination method using the synthetic primers. The sequences of the three primers are as follows:

3604–3620 (5′ATTCACCTAAATCTAAT3′),

3974–3990 (5′GCTTTACCTTTATGATT3′),

4392–4376 (5′TGTTAATGGTCATGGAC3′).

(ii) *Sequence analyses*

The DNA sequence alignment was performed by GENETYX software version 5 and 8 (SDC Software Development). The synonymous substitutions were calculated by the method of Nei & Gojobori (1986). The corrections for multiple substitutions in a site were done by the method of Jukes & Cantor (1969). Amino-acid sequences were deduced from the DNA sequences. The alignment of the amino- acid sequences was done by the GENETYX software (version 5), and then by eye. The amino-acid substitutions per site were calculated under the model of Poisson process (Nei, 1987).

3. Results

(i) *Mitochondrial DNA sequence of* D. simulans *and* D. mauritiana

The sequence alignment of the 1990 bp mtDNA fragments of *D. simulans* and *D. mauritiana* is given in Fig. 1. The gene organization of the two sequences was identical to the sequences of *D. melanogaster* and *D. yakuba* (de Bruijn, 1983; Clary & Wolstenholme, 1985). Nucleotide substitutions between the two sequences determined were observed at 39 positions.

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Table 1. *Nucleotide substitutions (synonymous) of four mtDNA genes among* Drosophila melanogaster, D. simulans, D. mauritiana *and* D. yakuba

Species compared	No. of synonymous substitutions ($\times 10^{-1}$)			
	ND2	COI	COII	ATPase 6
mel-1 & mel-2	4 (0·25±0·13)	10 (0·29±0·09)	3 (0·21±0·12)	1 (0·07±0·07)
sim & mau	19 (1·29±0·30)	43 (1·35±0·21)	12 (0·90±0·26)	14 (1·00±0·27)
sim & mel-1	30 (2·15±0·41)	81 (2·79±0·33)	23·5 (1·88±0·40)	18 (1·31±0·32)
sim & mel-2	26·5 (1·86±0·37)	76 (2·59±0·31)	24·5 (1·97±0·41)	19 (1·39±0·33)
mau & mel-1	22 (1·51±0·33)	62 (2·04±0·27)	25 (2·01±0·42)	23 (1·72±0·37)
mau & mel-2	21 (1·43±0·32)	55 (1·78±0·25)	26 (2·11±0·43)	22 (1·64±0·36)
sim & yak	49 (3·91±0·61)	114 (4·32±0·45)	38 (3·32±0·58)	30·5 (2·38±0·45)
mau & yak	43 (3·31±0·54)	99 (3·59±0·39)	37 (3·21±0·57)	36·5 (2·96±0·52)
mel-1 & yak	39 (2·93±0·50)	100 (3·63±0·39)	38 (3·32±0·58)	36 (2·91±0·52)
mel-2 & yak	39 (2·93±0·50)	98 (3·54±0·39)	40 (3·54±0·61)	37 (3·01±0·53)

Each number within parentheses denotes the number of synonymous substitutions per site and the standard error ($\times 10^{-1}$). The number of synonymous sites for each gene compared is 160·8 for ND2, 347·3 for COI, 141·6 for COII, and 149·3 for ATPase 6.
Abbreviations: mel-1 and mel-2, two sequences of *D. melanogaster*; sim, *D. simulans*; mau, *D. mauritiana*; yak, *D. yakuba*.

When we compared this region among the four species, nucleotide substitutions were observed in 165 sites. In *D. simulans* and *D. mauritiana*, ATPase 8 genes start with the codon ATC immediately distal to the $tRNA^{Asp}$ gene. Although there is one ATA codon nine bases downstream from the codon ATC, comparison with *D. melanogaster* and *D. yakuba* sequences indicates that the codon ATC is most likely to be the initiation codon in these species. The ATPase 8 genes of *D. melanogaster* and *D. yakuba* start with the codon ATT, which is also unusual (de Bruijn, 1983; Clary & Wolstenholme, 1985). Initiation codon ATC has been found in several mtDNA sequences such as mouse, locust and *Paramecium* (Bibb *et al.* 1981; Haucke & Gellisen, 1988; Pritchard *et al.* 1990). Other initiation codons examined in this study are ATG in all the four species.

(ii) *The synonymous substitution rate*

For each pair of the four species, including the two strains of *D. melanogaster*, we have calculated the number of synonymous substitutions for each of the four protein-coding genes, ND2, COI, COII and ATPase 6 (Table 1). Although the number of synonymous substitutions per site for each pair of the species varies (by a factor of 2·1) depending on the gene compared, when the relationships between two species are closer, the number of synonymous substitutions is smaller. In the ATPase 6 gene, for example, the number of synonymous substitutions for the closest pair of species *D. simulans–D. mauritiana* is 14. The number is 18 for *D. melanogaster–D. simulans* and 23 for *D. melanogaster–D. mauritiana* which are more distantly related species and 30·5 to 37 for the most distantly related pairs of species, *D. yakuba* and the three species of the *melanogaster* complex. For the other three genes, a similar tendency was also observed. This result is inconsistent with Sharp & Li (1989) who have reported that the level of synonymous substitution in the COI gene of *D. simulans–D. mauritiana* is higher than that of *D. melanogaster–D. simulans*. This might be because they used only a small number of codons for the estimation of the number of synonymous substitutions.

(iii) *The amino-acid substitution rate*

The amino-acid substitutions are summarized in Table 2. In COI and COII, the numbers of amino-acid substitutions are so small that it is not clear whether the amino-acid substitution rate is constant or not. Although the amino acid sequences of ATPase 6 in *D. simulans*, *D. mauritiana*, and *D. yakuba* are not so

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Table 2. *Amino-acid substitutions of the four mtDNA genes among* D. melanogaster, D. simulans, D. mauritiana *and* D. yakuba

Species compared	No. of amino-acid substitutions ($\times 10^{-2}$)			
	ND2	COI	COII	ATPase 6
mel-1 & mel-2	2 (0·72 ± 0·51)	0 (0·00 ± 0·00)	0 (0·00 ± 0·00)	3 (1·35 ± 0·78)
sim & mau	5 (1·82 ± 0·81)	1 (0·20 ± 0·20)	3 (1·32 ± 0·76)	0 (0·00 ± 0·00)
sim & mel-1	14 (5·19 ± 1·39)	4 (0·79 ± 0·39)	5 (2·22 ± 0·99)	7 (3·17 ± 1·20)
sim & mel-2	13 (4·81 ± 1·33)	4 (0·79 ± 0·39)	5 (2·22 ± 0·99)	4 (1·80 ± 0·90)
mau & mel-1	12 (4·43 ± 1·28)	3 (0·59 ± 0·34)	3 (1·32 ± 0·76)	7 (3·17 ± 1·20)
mau & mel-2	10 (3·68 ± 1·16)	3 (0·59 ± 0·34)	3 (1·32 ± 0·76)	4 (1·80 ± 0·90)
sim & yak	18 (6·72 ± 1·58)	3 (0·59 ± 0·34)	3 (1·32 ± 0·76)	3 (1·35 ± 0·78)
mau & yak	15 (5·57 ± 1·44)	4 (0·79 ± 0·39)	3 (1·32 ± 0·76)	3 (1·35 ± 0·78)
mel-1 & yak	18 (6·72 ± 1·58)	5 (0·98 ± 0·44)	4 (1·77 ± 0·88)	8 (3·64 ± 1·29)
mel-2 & yak	16 (5·95 ± 1·49)	5 (0·98 ± 0·44)	4 (1·77 ± 0·88)	5 (2·26 ± 1·01)

Each number within parentheses denotes the number of amino-acid substitutions per site and standard error ($\times 10^{-2}$). The number of amino-acid sites for each gene compared is 277 for ND2, 511 for COI, 228 for COII and 224 for ATPase 6.
Abbreviations of the strains are as given in Table 1.

Table 3. *Polymorphic sites among four sequences of* D. melanogaster

Strain	Amino-acid position				
	12	62	177	180	187
mel-1	L (TTA)	G (GGA)	N (AAT)	S (TCT)	M (ATA)
mel-2	S (TCA)	G (GGT)	N (AAT)	P (CCT)	V (GTA)
SP-1	S (TCA)	G (GGT)	K (AAA)	S (TCT)	M (ATA)
IR-17	S (TCA)	G (GGT)	N (AAT)	P (CCT)	V (GTA)

Only the polymorphic sites are shown. The codon corresponding to each of the amino-acid residues is in parentheses.

divergent from one another, it appears that the rate of amino-acid substitutions in comparison with one of the *D. melanogaster* sequences from de Bruijn (1983) (mel-1) is relatively higher than with the other three species. In contrast, the substitution rate in ND2 seems to be constant.

A χ^2 test has been performed to see if the amino-acid substitution rate is higher in *D. melanogaster* ATPase 6 than in other genes or in other strains. When we use one of the two *D. melanogaster* sequences (mel-1), the number of amino-acid substitutions in ATPase 6 is significantly larger than in the other three species ($\chi^2 = 7{\cdot}4$, $P < 0{\cdot}025$, $\nu = 2$). However, when the other strain is used, it is not significant ($\chi^2 = 1{\cdot}46$, $P > 0{\cdot}25$, $\nu = 2$).

(iv) *The polymorphisms in the* D. melanogaster *ATPase 6*

Three amino-acid substitutions were found between the two ATPase 6 genes of *D. melanogaster* (Table 2). To examine the degree of polymorphism, the ATPase 6 genes of another two *D. melanogaster* strains (SP-1, IR-17) were sequenced. These DNA sequences and the two sequences from de Bruijn (1983) were different from one another except that mel-2 and IR-17 were identical, indicating a high degree of sequence polymorphism. There are five polymorphic nucleotide sites of which four are nonsynonymous (Table 3). The average number of nucleotide differences per site between two sequences is $4{\cdot}48 \times 10^{-3}$.

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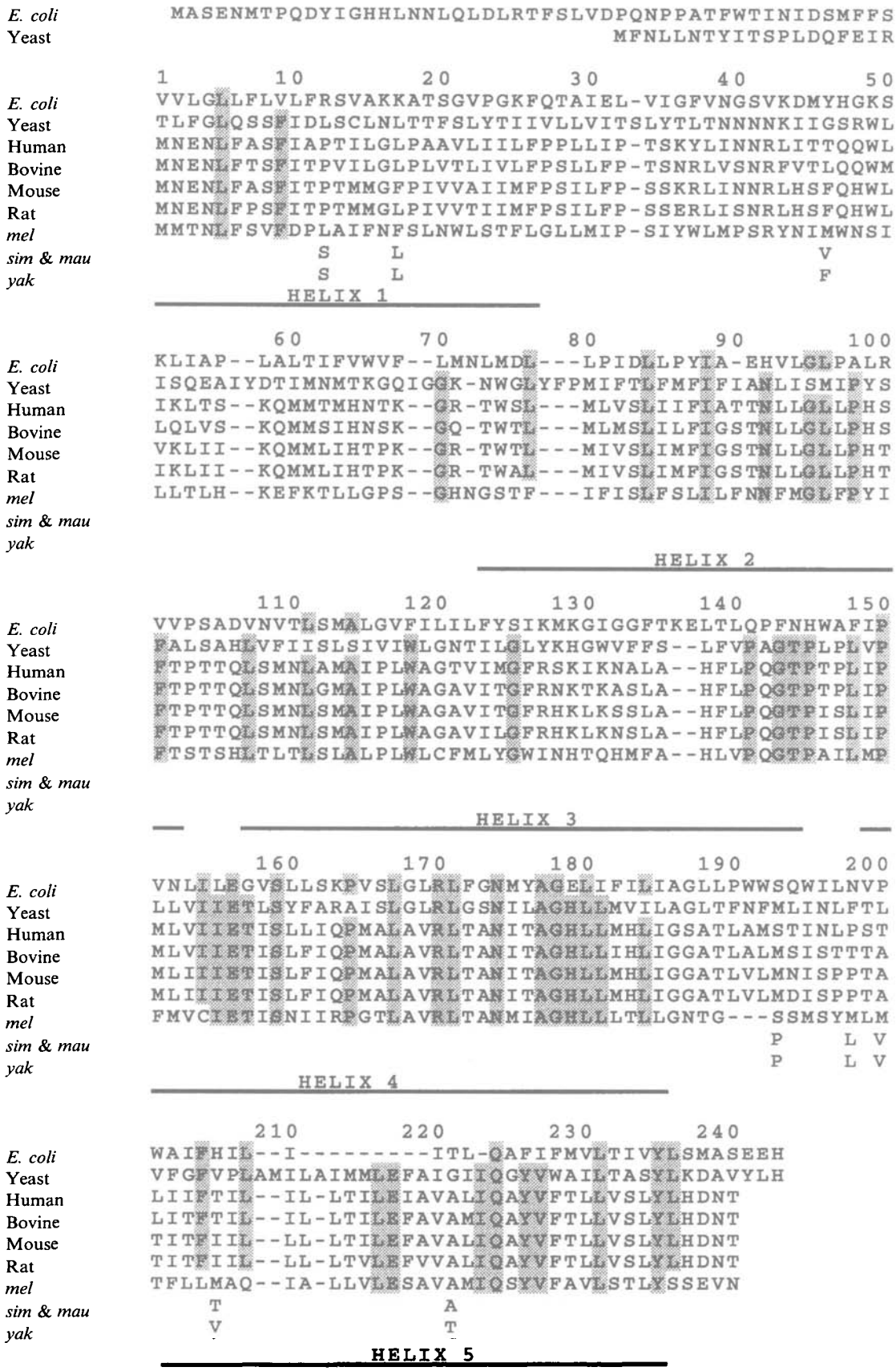

Fig. 2. Amino-acid sequences of *Drosophila* ATPase 6 compared with the counterparts encoded in *E. coli unc* operon (Walker *et al.* 1984), and yeast (John & Nagley, 1987), bovine (Anderson *et al.* 1982), mouse (Bibb *et al.* 1989), human (Anderson *et al.* 1981), and rat (Grosskopf & Feldman, 1981) mtDNA. Only one sequence of *D. melanogaster* (mel-1 in Table 1, Table 2, and Fig. 2) is shown as the sequence of *Drosophila*. Differences from this sequence in *D. simulans*, *D. mauritiana*, and *D. yakuba* are shown below the sequence of *D. melanogaster*. The sites where amino-acid residues are conserved among more than six organisms are shaded. Because of deletions and insertions among different species, amino-acid position indicated on the top line is different from Table 3. Lines below the sequences represent the regions of the transmembrane helices deduced from the protein sequence of human ATPase 6 by Cox *et al.* (1986).

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4. Discussion

The present study demonstrates that the amino-acid substitution rate of the mitochondrial gene, ATPase 6, is higher in some strains of *D. melanogaster* than in three other species of the *melanogaster* subgroup of *Drosophila*. We also found intraspecific variation in ATPase 6 of *D. melanogaster*. Most of interspecific differences between *D. melanogaster* and the other species were found in the polymorphic sites within *D. melanogaster*. High mutation rate could not cause the polymorphism in the *D. melanogaster* ATPase 6, because synonymous substitution rate in this gene is almost constant among the four species examined. We suppose that these polymorphisms are not maintained by positive selection nor have been fixed in the population of *D. melanogaster*. If the mutations are positively selected, they would be fixed rapidly unless certain mechanism such as overdominant selection maintains the polymorphisms just as in major histocompatibility complex (MHC) (Hughes & Nei, 1988, 1989). The mutations in the ATPase 6 gene, however, may be nearly neutral, and the loss of selective constraint may cause the change of amino-acid substitution rate.

If we assume the hypothesis above, the change in the extent of selective constraint against the ATPase 6 protein might be related to the change of the substitution rate. In order to see whether the sites at which amino-acid substitutions were observed are conserved among various organisms, we compared the amino-acid sequences of ATPase 6 proteins of the four species of *Drosophila* with its counterparts of *E. coli* (Walker *et al.* 1984), yeast (John & Nagley, 1987), and four mammals; human (Anderson *et al.* 1981), bovine (Anderson *et al.* 1982), mouse (Bibb *et al.* 1981), and rat (Grosskopf & Feldmann, 1981) (Fig. 2). Although the homology is relatively weak in the N-terminal region, these sequences are clearly homologous. Strong homology was observed in transmembrane helices 4 and 5 which are involved in the proton translocation and supposed to be interacting with subunit 9 (Cox *et al.* 1986; Nagley, 1988; Hoppe & Sebald, 1986). The homology appears relatively weak in the positions where the amino-acid substitutions were observed among the four species (Fig. 2). However, most of these positions are still conserved between rat and mouse, which are supposed to have diverged 20–29 Mya (O'hUigin & Li, 1992). There might be still functional constraints in these positions to some extent in *D. simulans*, *D. mauritiana* and *D. yakuba*, but lost in *D. melanogaster*.

The effect of amino-acid substitutions observed in *D. melanogaster* can be considered from the viewpoint of the character of each amino acid residue. The amino-acid positions which differ solely in ATPase 6 of *D. melanogaster* from the other three species are in helix 1 and the loop between helices 4 and 5 (Fig. 2). Of the two amino acid substitutions in helix 1, one is between a hydrophobic residue, L, and a hydrophilic residue, S (position 12 in Fig. 2), and the other is between two hydrophobic residues L and F (position 17). Since helix 1 is in the lipid bilayer of the membrane, the substitution at position 12 from S to L which retains hydrophobicity may be conservative. Among the three amino-acid substitutions in the region between helices 4 and 5, two were conservative changes between hydrophobic residues, but the third was from P to S (position 193 in Fig. 2), that is from hydrophobic to hydrophilic. Moreover, P is likely to be a breaker of α-helix (Chou & Fasman, 1978). For these reasons, the P to S substitution may be nonconservative. In addition, K to N substitution was observed when SP-1 was used as the strain of *D. melanogaster* in the comparison. This substitution may be also nonconservative because K is a basic amino acid and N is a non-polar hydrophilic amino acid. The clustering of the amino-acid substitutions and lack of conservation in some of the sites in the region between the helices 4 and 5 where the differences were observed indicate that some functional or conformational change in the mitochondrial H^+-ATPase of *D. melanogaster* could cause the decrease of the constraint in those regions.

Finally, we should point out that high degrees of mtDNA polymorphism have been observed in some species of the *melanogaster* subgroup (Solignac *et al.* 1986). These polymorphisms appear to be older than the divergence time of two closely related species, and are thought to be maintained by the imperfect maternal inheritance of mtDNA. It would be interesting, therefore, to examine the polymorphisms in the ATPase 6 gene of the other species of the *melanogaster* subgroup.

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