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MOLECULAR VARIATION FOR *mtDNA* HAPLOTYPES OF *Drosophila melanogaster* AND *D. simulans* NATURAL POPULATIONS BASED ON MORE ACCURATE RESTRICTION-SITE MAP

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ABSTRACT

Four restriction enzymes; *Hae* III, *Msp* I (4-base cutters), *Pvu* II and *Sal* I (6-base cutters), were used to reconstruct restriction-site map, with better resolution, for the mtDNA from nine geographically different natural populations of *D. melanogaster* and *D. simulans*. A total of 32 restriction sites were scored for mtDNA haplotypes; 24 sites of them were from *D. melanogaster* and the remaining eight sites were from *D. simulans*. Five restriction sites were found to be shared between the two species. Six fragment patterns were scored for each of *Hae* III and *Msp* I and two fragment patterns were scored for each of *Pvu* II and *Sal* I. In *D. simulans*, only one fragment pattern was scored for each of the four restriction enzymes. Eight sites (*Hae* III, 0.8, *Msp* I, 1.0, *Pvu* I, 4.1, 7.1 and 11.0, and Sal I, 2.0, 7.0 and 14.8) were detected in all *D. melanogaster* mtDNA haplotypes and were considered to be monomorphic. The highest estimate of polymorphism (94.29%) was detected for each of the two sites of *Hae* III, 4.4 and 10.6. The highest significant estimates of polymorphism were recorded for each of Alexandria (0.0356±0.0012), Baltim (0.0344±0.0), Beni-Sueef (0.0338±0.0018), and Assiut (0.0325±0.023) haplotypes. The lowest estimate of polymorphism was recorded for Sallom haplotypes (0.1250±0.0). The highest estimate of heterozygosity (*H*) was recorded for Alexandria haplotype (0.0230±0.0134). The net nucleotide divergence rates (d_A) ranged from 0.0014 (between the two *D. simulans* haplotypes) to 0.1415 (between Ismailia, *D. simulans* and Alexandria, *D. melanogaster*). The highest estimates of (d_A) between *D. melanogaster* haplotypes were recorded between Sallom and each of Beni-Sueef (0.0509) and Assiut (0.0504). Both methods of phylogenetic tree, UPGMA and NJ joined together the two populations of *D. simulans* in one cluster apart from *D. melanogaster* with a branch length equal to 0.1263. The NJ tree used Assiut population as a root. There were fifty cases (10 significant and 40 highly significant) of disequilibria between the 496 combinations of the 32 scored sites with percentage of 10.08%.

Keywords: Molecular population genetics, molecular evolution, mitochondrial DNA, *Drosophila melanogaster*, *D. simulans*, nucleotide diversity, gametic disequilibrium, phylogenetic trees.

INTRODUCTION

Determining the relative contributions of different microevolutionary processes in the maintenance of genetic diversity in natural populations has been a major focus in evolutionary biology for several decades. The advent of allozyme electrophoresis in the 1960s (Harris, 1966; Lewontin and Hubby, 1966) and the burgeoning amount of DNA information prompted a proliferation of studies that examined genetic diversity across a broad taxonomic spectrum (Piertney and Oliver, 2006).

An extensive literature exists on the patterns of genetic variation within and among populations of *Drosophila melanogaster* (Megeed *et al.*, 1998a). *Drosophila melanogaster* has a molecular signature of population

expansion from Africa (Hale and Singh, 1991 and Aquadro *et al.*, 2001) spread to Europe and Asia, and is believed to have colonized the New World in the past few hundred years. Population samples from Africa show abundant genetic variation, while samples from non-African populations carry a subset of that variation (Begun and Aquadro, 1995; Moriyama and Powell, 1996). Levels of genetic variation are typically reduced in New World populations, consistent with a founder event (Townsend and Rand, 2004).

Different studies to date have pointed practically to all the population mechanisms that can change gene frequencies (cytonuclear coadaptation, direct natural selection on mtDNA and random genetic drift) as being forces potentially acting on the mtDNA haplotypes (Christie *et al.*, 2004). An important difference between the effects of natural selection and history on genetic variation is that natural selection will exert its influence differently on individual loci, while the influence of history should be more or less equivalent on all loci.

In *D. simulans*, mtDNA genotypes were found to affect their fitness (James *et al.*, 2003). Flies with specific haplotype were found to be the fastest in developing and have the lowest probability of surviving and wild-type males with that mtDNA haplotype were found to be more active.

Results indicated that the maintenance of the frequencies of mtDNA haplotypes in natural populations could be due to their association with chromosomal arrangements (Megeed *et al.*, 1998b and Oliver *et al.*, 2005).

The most effective means of understanding population structure is to compare patterns of genetic variation with multiple genetic markers. Quantification of the population structure of mtDNA length variation should provide focused information on recent population history.

In this paper, restriction analysis of mtDNA polymorphism was used to infer species history in *D. melanogaster* and *D. simulans*. In a preliminary report (Megeed *et al.*, 1998a), natural populations of *D. melanogaster* showed a much greater degree of population structure for mtDNA restriction haplotypes using two restriction enzymes. Here, that analysis will be extended by means of increasing number of enzymes, and resolution of the full restriction-site map.

MATERIALS AND METHODS

I- Collection localities of *Drosophila* natural populations:

Seven natural populations of *D. melanogaster* and two of *D. simulans* were collected from different geographic localities in Egypt. The flies were collected at approximately 200-600 kilometers intervals such as Sinai, Suez Canal, Nile Delta, North Coast, Upper Egypt and Western Desert. These localities and their abbreviations were as follows:

***D. melanogaster* populations:**

1-Aresh (AR), 2- Ismailia (IS), 3-Baltim (BA), 4-Alexandria (AL), 5-Sallom (SA), 6- Beni-Sueef (BS), and 7- Assiut (AS).

***D. simulans* populations:**

1- Ismailia (ISs) and **2-** Bowaty (BOs), the Western Desert.

Five iso-female lines were constructed for each population of the two species to give a total number cf 45 strains, which were incubated at 25°C in the laboratory. For each natural population, a single female was put together with three to five males to initiate the iso-female line. Each iso-female line was transferred to a new vial each generation for at least 30 generations. The optimal medium (cornmeal, agar, molasses, water and propionic acid) was used.

II - Isolation of mitochondrial DNA (mtDNA):

Rapid isolation method of Tamura and Aotsuka (1988) was used with some modifications as previously described (Megeed *et al.*, 1998a).

III- Digestion with restriction enzymes and agarose gel electrophoresis

Four restriction endonucleases, *Hae* III, *Msp* I (4-base cutters), *Pvu* II and *Sal* I (6-base cutters), were used. These four enzymes and their buffers were obtained from Boehriger Mannheim Ltd. Co., Germany. The recoginition sequence of these enzymes are: GG^CC for *Hae* III, C^CGG for *Msp* I, CAG^CTG for *Pvu* II and G^TCGAC for *Sal* I. The reactions were assembled by mixing about 1 µg of mtDNA, 5 µl of 10 x buffer and 3-5 units of the enzyme in a sterile eppendorf tube, according to Sambrook *et al.* (1989). Sterile bi-distilled water was added to a final volume of 50 µl. The reactions were incubated at 37°C for 3 h, 5 µl of stop solution (0.05% bromophenol blue, 50 mM EDTA and 0.05 % SDS) were added to stop the reaction.

The DNA samples were loaded on 0.8% agarose gels, with TBE running buffer (Sambrook *et al*, 1989). After electrophoresis, the gels were stained with ethidium bromide (0.5 µg/ml) for 15 min and destained with water for 15min, and mtDNA bands were visualized under UV trans-eleminator plate. Gels were photographed using Polaroid camera DS34. Sizes of restriction fragmen'ts were estimated by a graphical method using *Hind* III digested lambda phage DNA as a size marker.

IV-Data analysis

Polymorphism was estimated for each mtDNA haplotype according to Weir (1990) as: $p = k / 2 j m$, where k = number of polymorphic sites, j = average of the two recognition sequence lengths and m = total number of sites. Polymorphism was calculated for each natural population as the average over its five haplotypes.

Estimates of nucleotide diversity (nucleotide substitution rate; d_{ij}, the average of the number of nucleotide substitutions per site between two sequences), the net nucleotide divergence rate (d_A) and nucleotide diversity (π) were calculated according to Nei (1987) and Nei and Miller (1990) as follows: $d_{ij} = -3/4 \log_e (1 - (4p_{ij} / 3))$, *where* p_{ij} *is the average of the proportion of different nucleotides between two sequences over all pair wise comparisons,* $d_A = d_{xy} - (d_x + d_y) / 2$, *where* d_x *is the nucleotide substitution rate of the population (x) and* d_y *is the nucleotide substitution rate of the population (y) and*

$$\pi = 2 \sum_{i<j} d_{ij} / n(n-1)$$

The restriction-site maps for mtDNA in the 45 iso-female lines were used to calculate the nucleotide sequence divergence between each pair of lines, according to Nei (1987). The matrix of genetic distances was used to

generate phylogenetic trees by means of Saitou's and Nei's (1987) and the use of two different computer software programs "Restsite" and "Tdraw" (Nei and Miller, 1990).

Linkage disequilibrium was estimated according to Weir (1990):
$D_{AB} = p_{AB} - p_A p_B$, *where* p_i *is the frequency of different restriction sites and i* =*A, B or AB.* with variance equal to:
$Var(D_{AB}) = 1/n[p_A(1-p_A)\,p_B(1-p_B) + (1-2p_A)(1-2p_B)\,D_{AB} - D^2_{AB}]$
, and chi square test was used as: $\chi^2_{AB} = nD^2_{AB} / p_A(1-p_A)\,p_B(1-p_B)$

The time of divergence between *D. melanogaster* populations or between *D. melanogaster* and *D. simulans* was estimated according to Nei (1987) as: $\pi = 2\lambda T$, assuming a constant rate of nucleotide substitution, *where* π *= number of nucleotide diversity between two species,* λ*= rate of nucleotide substitution, and T = time since divergence.*

RESULTS AND DISCUSSION

1- Sizes and patterns of restriction fragments:

The total size of mtDNA was estimated to be 18.8 and 18.9 kbp in *D. melanogaster* and *D. simulans*, respectively. These sizes are similar to those reported before by Megeed *et al.*, (1998a) with slight difference to 18.6 and 18.9 kbp which were reported by Shah and Langley (1979) and Choi and Choo (1993), respectively in *D. melanogaster*. Solignac and Monnerot (1986) estimated mtDNA size of *D. simulans* to be 18.9 kbp while the estimation of Choi and Choo (1993) was 19.0 kbp. This size variation may be due to cases of insertion or deletion and, also, to the presence of transposable genetic elements.

The basic restriction-site maps for the mtDNA in each of *D. melanogaster* and *D. simulans* are presented in Figure (1). The previous results of Megeed *et al.* (1998a) were used in this investigation to reconstruct a better restriction-site map and recalculate the nucleotide substitution rates between the two species and their natural populations.

A total of 32 restriction sites were scored for mtDNA haplotypes of *D. melanogaster* (24 restriction sites) and *D. simulans* (eight restriction sites). Five restriction sites; *Hae* III, 0.8; *Msp* I, 1.0; *Pvu* II, 4.1, *Sal* I, 7.0 and *Pvu* II, 7.1 were found to be shared between the two species. Figure (2) shows representative examples for mtDNA digests with *Hae* III, *Msp* I, *Pvu* II and *Sal* I and their different patterns.

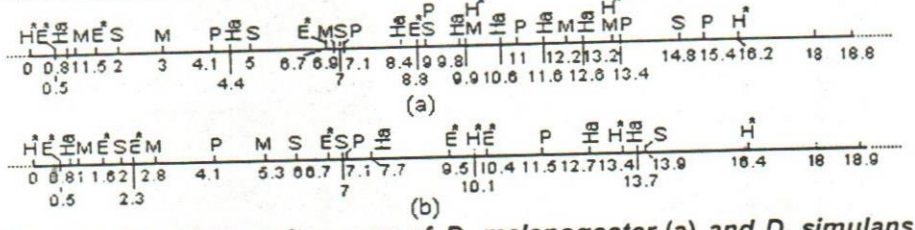

Figure 1. Restriction site maps of *D. melanogaster* (a) *and D. simulans* (b) mtDNA.
***E = *Eco* RI, H = *Hind* III (Megeed *et al.*, 1998a), Ha = *Hae* III, M = *Msp* I, P = *Pvu* II and S = *Sal* I.**

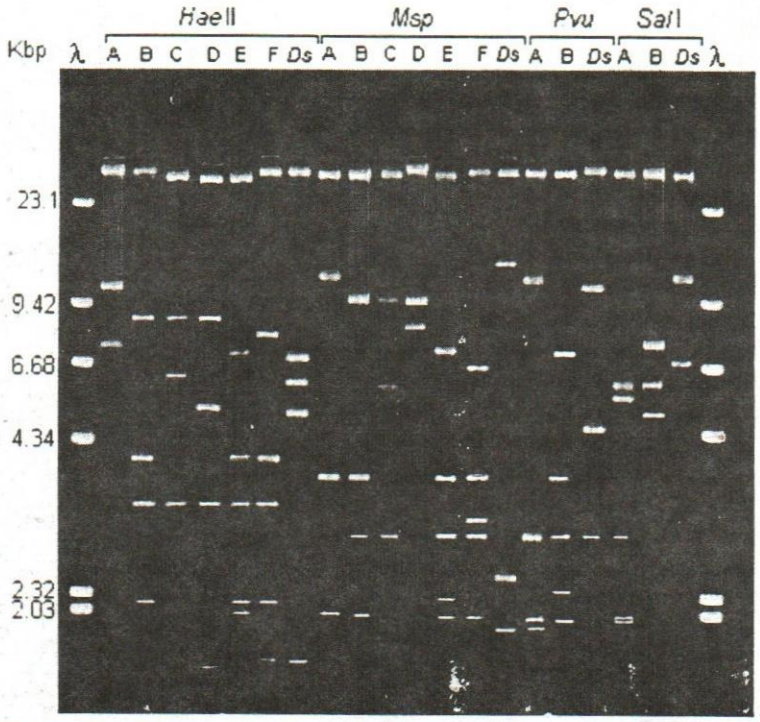

Figure 2. Agarose gel electrophoresis for mtDNA in *D. melanogaster* and *D. simulans* (*Ds*) natural populations with different patterns of *Hae* III, *Msp* I, *Pvu* II and *Sal* I digest.

Table 1. Fragments patterns of *D. melanogaster* and *D. simulans* mtDNA.

Enzyme	*Hae* III							*Msp* I						
Species	*D. melanogaster*						*D. simulans*	*D. melanogaster*						*D. simulans*
Patterns	A	B	C	D	E	F	G	A	B	C	D	E	F	G
Fragment size (kbp)	11.2 7.6	9 4 3.6 2.2	9 6.2 3.6	9 5.4 3.6 0.8	7 4 3.6 2.2 2	8 4 3.6 2.2 1	6.9 6 5 1	12.9 3.9 2	9.9 3.9 3 2	9.9 5.9 3	9.9 8.9	7.6 3.9 3 2.3 2	6.6 3.9 3.3 3 2	14.6 2.5 1.8

Enzyme	*Pvu* II			*Sal* I		
Species	*D. melanogaster*		*D. simulans*	*D. melanogaster*		*D. simulans*
Patterns	A	B	C	A	B	C
Fragment size (Kbp)	11.9 3 2 1.9	7.5 3.9 3 2.4 2	11.5 4.4 3	6 5.8 3 2 2	7.8 6 5	12 6.9

Sixteen different fragment patterns were determined for the *D. melanogaster* mtDNA digest using four restriction enzymes as shown in Table (1) and Figure (3). For each of *Hae* III and *Msp* I six fragment patterns were scored and two fragment patterns were scored for each of *Pvu* II and *Sal* I. In *D. simulans*, only one fragment pattern was scored for each of the four restriction enzymes.

2- Restriction-site map variation:

Eight sites (*Hae* III, 0.8, *Msp* I, 1.0, *Pvu* I, 4.1, 7.1 and 11.0, and Sal I, 2.0, 7.0 and 14.8) were detected in all *D. melanogaster* mtDNA haplotypes and were considered to be monomorphic. Five of these sites were found to be shared with *D. simulans*; *Hae* III; 0.8, *Msp* I; 1.0, *Pvu* II; 4.1 and 7.1 and *Sal* I; 7.0. Shah and Langely (1979) considered these sites as conserved ones in both *D. melanogaster* and *D. simulans* mtDNA. Also the sites of *Sal* I; 2.0 and *Hae* III; 11.0 may be considered as conserved sites since they were shared by all *D. melanogaster* natural populations.

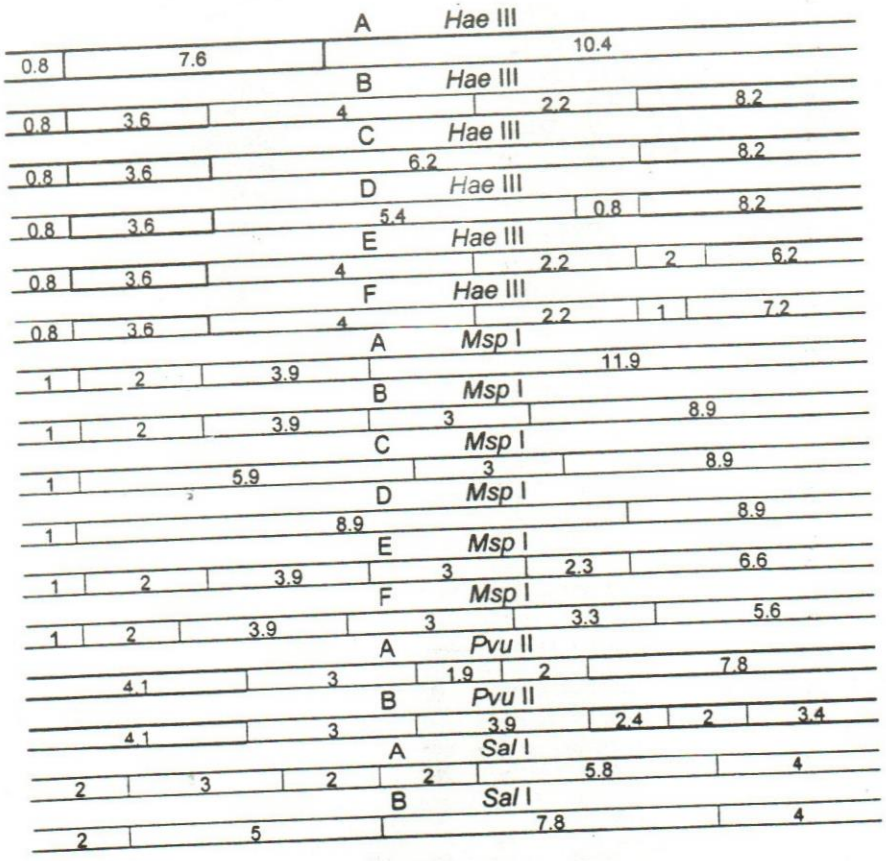

Figure 3. Restriction patterns for mtDNA of *D. melanogaster*.

Table (2) presents the estimates of polymorphism in sixteen polymorphic sites in *D. melanogaster.* The highest estimates of polymorphism (94.29%) were detected for each of the two sites of *Hae* III, 4.4 and 10.6, whereas the lowest estimate (5.71%) was detected for the site *Hae* III, 11.6. The scored polymorphism for the other sites was found to be ranged from as low as 5.71% (*Pvu* II, 11.0) to 85.71% (*Msp* I, 6.9 and *Hae III*, 9.8).

The estimates of polymorphism for each natural population with their standard errors and their ANOVA analysis are presented in Table (3). The highest significant estimate was recorded for each of Alexandria (0.0356±0.0012), Baltim (0.0344±0.0), Beni-Sueef (0.0338±0.0018), and Assiut (0.0325±0.023) haplotypes. The lowest estimate of polymorphism was recorded for Sallom haplotypes (0.0125±0.0).

Table 2. Estimates of polymorphism (as percentage) in sixteen polymorphic sites in *D. melanogaster* mtDNA.

Sites	M 3.0	Ha 4.4	S 5.0	M 6.9	Ha 8.4	P 9.0	S 9.0	Ha 9.8	M 9.9	P 11.0	Ha 11.6	M 12.2	Ha 12.6	M 13.2	S 14.8	P 15.4
Polymorphism %	74.29	94.29	54.29	85.71	51.43	45.71	22.86	85.71	94.29	5.71	34.29	11.43	20	54.29	57.14	22.86

Table 3. Estimates of polymorphism for seven mtDNA haplotypes in *D. melanogaster* mtDNA and their mean square estimates.

mtDNA Haplotypes	Polymorphism	ANOVA			
		Source	d.f.	MS	F
1-Aresh	0.0175±0.0008bc	Haplotypes	6	0.000476	55.88**
2-Ismailia	0.0188±0.0009b				
3-Baltim	0.0344±0.0a				
4-Alexandria	0.0356±0.0012a				
5-Sallom	0.0125±0.0c				
6-Beni-Sueef	0.0338±0.0018a	Error	28	0.0000852	
7-Assiut	0.0325±0.0023a				
LSD (0.01)	0.0051				

The presence (+) and absence (-) matrix of mtDNA restriction sites of the 45 different iso-female lines was used to generate estimates of nucleotide substitution rates (d_{ij}) between these iso-female lines, then the net nucleotide divergence rate (d_A) was calculated. Estimates of (d_A) between natural populations of *D. melanogaster* and *D. simulans* with heterozygosity (*H*), for each population on the diagonal, are shown in Table 4.

The highest estimate of heterozygosity (*H*) was recorded for the Alexandria population (0.0230±0.0134) and was close to that of polymorphism (0.0356±0.0012) for the same population. The other estimates of (*H*) were found to be ranged from 0.0 (Bowaty and Ismailia, *D. simulans*) to 0.0137 (Assiut, *D. melanogaster*). The lower estimates of (*H*) for Sallom

(0.0052), Baltim (0.0052), Ismailia (0.0057) and Aresh (0.0092) populations indicate the nature of environmental isolation and the effective size of population is very small.

The net nucleotide divergence rates (d_A) ranged from 0.0014 (between the two *D. simulans* haplotypes) to 0.1415 (between Ismailia, *D. simulans* and Alexandria, *D. melanogaster*). The highest estimates of (d_A) between *D. melanogaster* haplotypes were recorded between Sallom and each of Beni-Sueef (0.0509) and Assiut (0.0504). This isolated population of Sallom exhibited higher levels of nucleotide divergence with Baltim (0.0486) and Alexandria (0.0478) populations. This indicates that Sallom population is a very recent one and was recently diverged. Also, the rate of nucleotide substitution of mtDNA in this population is very slow compared to the older populations of Beni-Sueef and Assiut. Hale and Singh (1991), Begun and Aquadro (1995), Moriyama and Powell (1996), Aquadro *et al.* (2001) and Townsend and Rand (2004) stated that *D. melanogaster* originated from Africa (Old World) and spread to the New World, and that the African populations exhibited abundant genetic variations.

Table 4. Estimates of nucleotide divergence rate (± S.E.), below the diagonal, and heterozygosity (*H*), on the diagonal, for mtDNA between different natural populations of *D. melanogaster* and *D. simulans*.

	Populations								
	AR	IS	BA	AL	SA	BS	AS	ISs	BOs
AR	0.0092± 0.0064								
IS	0.0098± 0.0070	0.0057± 0.0049							
BA	0.0399± 0.0129	0.0364± 0.0123	0.0052± 0.0048						
AL	0.0351± 0.0121	0.0317± 0.0115	0.0193± 0.0085	0.0230± 0.0134					
SA	0.0150± 0.0080	0.0214± 0.0150	0.0486± 0.0145	0.0478± 0.0151	0.0052± 0.0045				
BS	0.0391± 0.0135	0.0356± 0.0131	0.0099± 0.0062	0.0174± 0.0087	0.0509± 0.0157	0.0133± 0.0110			
AS	0.0369± 0.0132	0.0326± 0.0129	0.0165± 0.0109	0.0164± 0.0076	0.0504± 0.0154	0.0135± 0.0099	0.0137± 0.0102		
ISs	0.1227± 0.0432	0.1241± 0.0439	0.1301± 0.0471	0.1415± 0.0516	0.1177± 0.0349	0.1322± 0.0469	0.1282± 0.0445	0.0±0.0	0.0014± 0.0014
BOs	0.1208± 0.0428	0.1217± 0.0436	0.1280± 0.0471	0.1393± 0.0515	0.1156± 0.0344	0.1300± 0.0467	0.1260± 0.0443	0.0014± 0.0014	0.0±0.0

The net nucleotide diversity (π) for mtDNA in *D. melanogaster* was found to be equal to 0.0297± 0.0116 in this study. This estimate is four times than that of Hale and Singh (1991); 0.0053. The nucleotide diversity of mtDNA for *D. pseudoobscura* and *D. subobscura* (Afonso *et al.*, 1990 and Rozas *et al.*, 1990), were 0.012 and 0.008; respectively. The nucleotide diversity of mtDNA for *D. immigrans* was 0.009. (Aotsuka *et al.*, 1994). The

nucleotide diversity (π) for nuclear genes such as *Adh* (Aquadro *et al.*, 1986), *rosy* gene region (Aquadro *et al.*, 1988) and *Notch* gene region (Schaeffer *et al.*, 1988) in *D. melanogaster* were 0.024, 0.003 and 0.005; respectively. These results indicate that the level of genetic variation in mtDNA is equal to that in some nuclear genes (*Adh*). Rand *et al.* (1994 and 2006) showed that interspecific mtDNA strains of *D. melanogaster* exhibited a wide range of variation. There is also evidence that mtDNA sequences may diverge (evolve) faster than many nuclear sequences (Brown *et al.*, 1982).

The low degree of variability in mtDNA of *D. simulans* is very consistent with the other estimates reported by many authors (Solignac and Monnerot, 1986; Baba-Aissa *et al.*, 1989; and Hale and Singh, 1991). Baudry *et al* (2006) found that an eastern group of *D. simulans* populations from continental Africa and Indian Ocean islands (Kenya, Tanzania, Madagascar and Mayotte island) is widespread, shows little differentiation, and has probably undergone demographic expansion.

The nucleotide differences between *D. melanogaster* and *D. simulans* populations were very high and ranged from 0.1156 with (SA vs. BOs) to 0.1415 with (AL vs. ISs). The AL population was the most distant population out of all the *D. melanogaster* populations from *D. simulans*. These results suggested that the substitution rate of *D. simulans* is much slower than *D. melanogaster* and the last one is more polymorphic. Gupta *et al.*, (1993) estimated the nucleotide differences between *D. bipectinata* and *D. melarkotliana* to be 0.0002.

Caccone *et al.* (1988) estimated the nucleotide sequence divergence per million years per lineage (λ) in *D. melanogaster* to be 0.85%. This estimate can be used to obtain an estimate for the time since divergence between *D. melanogaster* and *D. simulans* in the equation: $\pi = 2 \lambda T$. The estimated net nucleotide diversity (π) between *D. melanogaster* and *D. simulans* in this study was 0.1184. So the time since divergence (T) between these two species is 0.07×10^6 years ago.

3-Phylogenetic analysis:

Two different methods were used to study the phylogenetic relationships between different populations. The first method is the Unweighted Pair-Group Method with Arithmetic mean, UPGMA, (Sneath and Sokal 1973) (Figure 4). The tree clustered together in one group the two populations of *D. simulans*; ISs and BOs, since no mtDNA variation was obtained between the ten iso-female lines of *D. simulans*. The closest populations of *D. melanogaster* to *D. simulans* are SA, IS and AR populations. The tree shows that *D. simulans* is departed from *D. melanogaster*. The tree assigned SA to one branch then it joins AR with IS. The tree then joins the branch of SA with the cluster of IS and AR. This means that the nearest population to that cluster is SA.

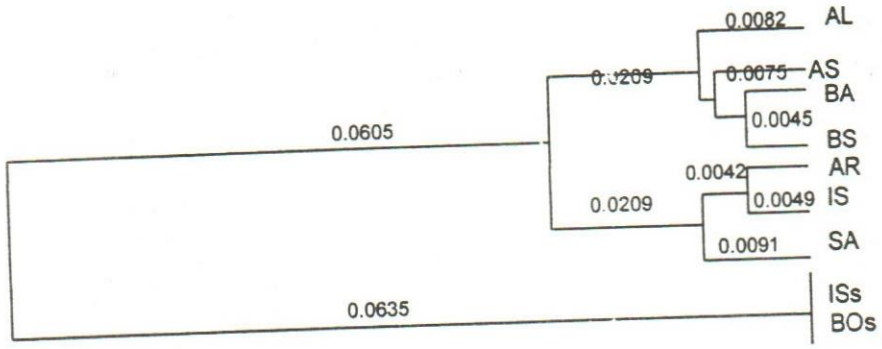

Figure 4. The UPGMA phylogenetic tree for mtDNA of *D. melanogaster* and *D. simulans* natural populations,

The tree and the (*d*) estimates proved that SA population is very isolated and diverged first from the *D. melanogaster / D. simulans* cluster. The next cluster was between BS and BA, which was grouped with AS population. At the last step, the tree joined Al with the last three populations.

The second method (Figure 5) is the Neighbor-Joining method, NJ, (Saitou and Nei 1987). The tree shows that AS is the oldest population and it was used as a root. The link between *D. melanogaster* and *D. simulans* was this population (AS). The tree also joined together the three populations of AR, IS and SA as the UPGMA method. The tree clustered the two populations of BS and BA in one separate group and placed AL population alone in a separate branch.

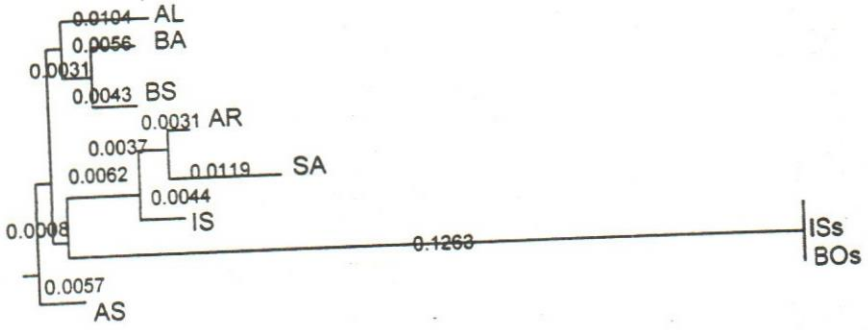

Figure 5. The NJ phylogenetic tree for mtDNA of *D. melanogaster* and *D. simulans* natural populations.

The phylogenetic tree generated by the NJ method is more reliable and widely used than the UPGMA method (Pearson *et al.*, 1999; Mailund *et al.*, 2006; Mueller, 2006 and Ogdenw and Rosenberg, 2006).

4-Linkage disequilibrium:

Pairwise comparisons were made between restriction sites on the mtDNA maps of the 35 studied isofemale lines of *D. melanogaster* to

determine the standardized coefficient of linkage disequilibrium ($D^{\backslash}$). This study used the results of both *Eco* RI and *Hind* III digests from a previous study (Megeed *et al.*, 1998a). Table 5 shows that there were 10 significant cases and 40 highly significant ones of disequilibria between the 496 combinations of the 32 scored sites (10.08%). Nine highly significant cases of $D^{\backslash}$ were detected between the site of *Msp* I, 3.0 and each of *Sal* I, 5.0; *Eco* RI, 6.7; *Msp* I, 6.9; *Pvu* II, 9.0; *Sal* I, 9.0; *Msp* I, 12.2; *Pvu* II, 13.4; *Pvu* II, 15.4 and *Hind* III, 16.2 (Table 5). Also, the site 5.0 of *Sal* I showed one case of significant $D^{\backslash}$ with *Eco* RI, 6.7 and seven cases of highly significant disequilibria with the sites *Msp* I, 6.9; *Pvu* II, 9.0; *Sal* I, 9.0; *Hae* III, 9.8; *Msp* I, 12.2; *Pvu* II, 13.4 and *Pvu* II, 15.4. On the other hand, the site Pvu II, 9.0 showed five highly significant cases of $D^{\backslash}$ and one significant one.

]Table 5. Estimates of significant linkage disequilibrium ($D^{\backslash}$) between restriction site mtDNA of *D. melanogaster* natural populations.

Sites	H 0.0	M 3.0	Ha 4.4	S 5.0	E 6.7	M 6.9	Ha 8.4	P 9.0
S 5.0		0.1396** ±0.0052						
E 6.7	0.0237* ±0.0041	0.0637** ±0.0400		0.0465* ±0.0041				
M 6.9		0.1061** ±0.0063		0.0775** ±0.0049	0.0735** ±0.0062			
Ha 8.4						0.0735* ±0.0047		
P 9.0		0.1396** ±0.0052		0.2196** ±0.0035	0.0465* ±0.0041	0.0653* ±0.0042		
S 9.0		0.1396** ±0.0052		0.2482** ±0.0012		0.0775** ±0.0049		0.2196** ±0.0035
Ha 9.8				0.10449** ±0.0048				0.0759* ±0.0053
Ha 10.6			0.0539** ±0.0059					
M 12.2		0.0882** ±0.0042	0.0375* ±0.0042	0.1575** ±0.0054				0.1281** ±0.0054
Ha 12.6							0.0555* ±0.0041	
M 13.2							0.0971** ±0.0049	0.0914** ±0.0047
P13.4		0.1396** ±0.0088		0.2196** ±0.0035		0.0775** ±0.0049		0.2482** ±0.0012
P 15.4		0.1469** ±0.0054		0.2326** ±0.0028				0.3102** ±0.0057
H16.2		0.6286** ±0.0097						

Sites	S 9.0	Ha 9.8	Ha 10.6	Ha 11.6	M 12.2	Ha 12.6	M 13.2	P13.4
Ha 9.8	0.1045** ±0.0048							
M 12.2	0.1567** ±0.0047	0.0931** ±0.0062	0.0375* ±0.0042					
M 13.2				0.0457** ±0.0050	0.0686* ±0.0039	0.0629** ±0.0058		
P13.4	0.2482** ±0.0012	0.0759* ±0.0053			0.12812** ±0.0054		0.0914** ±0.0047	
P 15.4	0.2612** ±0.0019				0.1469** ±0.0047			0.2326** ±0.0028
H16.2		0.0441** ±0.0048			0.0375* ±0.0042			

*** and ** means significance at *P* >0.05 and 0.01, respectively.**

These cases of (D') show that these sites are under selection which doesn't favor the presence together of these site-combinations. Mitochondria are often under strong selection and evolve under unusual evolutionary rules compared to other genomes (Ballard and Whitlock, 2004). Another explanation is that some of these sites are old which were detected in Assiut population, while others were detected in newer populations like Baltim (*Msp* I, 3.0; *Sal* I, 5.0; *Sal* I, 9.0; *Hae* III, 9.8; *Msp* I, 12,2; *Pvu* II, 13.4 and *Pvu* II, 15.4) and one site was detected in much newer and isolated populations like Saloom and Areesh (*Pvu* II, 9.0).

Because mtDNA is haploid and clonally inherited with no recombination, consequently, mutations accumulate over time within a clonal mtDNA line or haplotype. These mutations may become selectively advantageous and eventually will be fixed in that population. On the other hand, they become selectively disadvantageous in other environments or with other loci combinations.

The study succeeded in reconstructing restriction-site maps, with better resolution, for the nine mtDNA haplotypes of the two *Drosophila* species; *melanogaster* and *simulans*. These maps were used to reestablish the phylogenetic tree based on nucleotide substitution rates. There is still the need for using more restriction enzymes for reaching the best resolution of these maps.

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الإختلافات الجزيئية بين التراكيب الأحادية للحمض النووي الميتوكوندير ي لعشائر طبيعية *لدروسوفيلا ميلانوجاستر ودروسوفيلا سيمولانس* مبناة علي خريطة اكثر دقة لمواقع القطع

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استخدمت أربعة إنزيمات قطع ؛ *Hae* III ، *Msp* I (انزيمات قطع رباعية)، *Pvu* II و *Sal* I (انزيمات قطع سداسية)، لإعادة بناء خريطة أكثر وضوحا لمواقع القطع للحامض النووي الميتوكوندير ي في تسعة عشائر طبيعية مختلفة جغرافيا تابعة لنوعين من حشرة *الدروسوفيلا* هما *الميلانوجاستر والسيمولانس*. تم تحديد عدد ٣٢ موقع قطع في التراكيب الأحادية للحمض النووي الميتوكوندير ي (٢٤ من النوع *ميلانوجاستر* وثمانية للنوع *سيمولانس*). ووجدت خمسة مواقع قطع نبين أنها كانت مشتركة في كلا النوعين. ووجد أيضا ان هناك ستة أنماط للقطع المحرزة لكل من الإنزيمين *Hae* III و *Msp* I ونمطين لكلّ من *Pvu* II و *Sal* I . أما في النوع *سيمولانس* فقد تم احراز نمطا واحدا لكلّ منْ إنزيمات القطع الأربعة. وقد تم تحديد عدد ثمانية مواقع وجدت في كل التراكيب الأحادية للنوع *الميلانوجاستر* وهي : (*Hae* III, 0.8, *Msp* I, 1.0, *Pvu* I, 4.1, 7.1 and 11.0, and *Sal* I, 2.0, 7.0 and 14.8) وقد اعتبرت هذه التراكيب احادية الشكل المظهري . وتم تقدير أعلي نسبة مئوية لتعدد الأشكال المظهرية وكانت تساوي ٩٤,٢٩% لكلا الموقعين التابعين لإنزيم *Hae* III وهما ٤,٤ و ١٠,٦ . أما اعلي تقدير لحالات تعدد الأشكال المظهرية بالنسبة للتراكيب الأحادية فكانت (0.0356±0.0012) لعشيرة الإسكندرية و (0.0344±0.0) لعشيرة بلطيم و (0.0338±0.0018) لعشيرة بني سويف و (0.0325±0.023) لعشيرة اسيوط . أما أقل تقدير فكان يساوي (0.1250±0.0) لعشيرة السلوم . اما أعلي تقدير للخلط الوراثي فسُجّلَ للأسكندرية (0.0230 ±0.0134) . وتراوحت معدلات إنحرافِ النكليوتيداتِ (d_A) منْ ٠,٠٠١٤ (بين التراكيب الأحادية في النوع *السيمولانس* إلى ٠,١٤١٥ (بين التركيب الأحادي للإسماعيلية التابع لنوع السيمولانس والتركيب الأحادي لعشيرة الأسكندرية التابعة للنوع *الميلانوجستر*. وتم تسجيل أعلي التقديرات للـ (d_A) بين التراكيب الأحادية للنوع الميلانوجاستر بين السلوم وكلّ من بني سويف(0.0509) وأسيوط (0.0504). ضمت كلتا طريقتي شجر النسب الوراثي (UPGMA و NJ) عشيرتي النوع سيمولانس في فرع واحد متباعداً عن النوع ميلانوجاستر بفرع طوله ٠,١٢٦٣. إستعملتْ شجرةُ (NJ) عشيرة أسيوط كجذر. تم إيجاد خمسون حالة (عشرة حالات معنوية و٤٠ حالةِ تامة المعنوية) لعدم التوازن الارتباطي بين الـ٤٩٦ توفيقة للمواقع الـ ٣٢ المُحرَزة بنسبة مئوية قدرها ١٠,٠٨ %.