Mitochondrial DNA Evolution in the Montium-Species Subgroup of Drosophila

Pavlos Pissios and Zacharias G. Scouras

Department of Genetics, Aristotle University

Mitochondrial DNA (mtDNA) restriction-site maps for six species (10 strains) of the Drosophila montium subgroup were established. A total of 50 restriction sites were mapped, corresponding to 1.67% of the mtDNA genome. On the basis of differences in the restriction sites, nucleotide divergence (\(\delta\)) was calculated for each pair of species (strains), and phylogenetic trees were constructed by using distance-matrix and parsimony methods. Comparison of the resultant phylogenetic trees shows that the sibling species D. auraria and D. quadraria are closely related. At the other extreme, considerable divergence was observed between the two strains of D. serrata and between D. serrata and D. birchii, a finding that contrasts with their grouping within the same species complex. Nevertheless, our data indicate that these six oriental montium species are rather closely related.

Introduction

The Drosophila montium subgroup is the largest subgroup of the Drosophila melanogaster group and comprises 81 of the 156 known species of this group (for recent reviews, see Lemeunier et al. 1986; Ashburner 1989). Efforts have been made, using morphological, genetic, biochemical, and nuclear-DNA criteria, to establish phylogenetic relationships among different species of the montium subgroup [Ayala 1965; Bock and Wheeler 1972; Triantaphyllidis and Kastritsis 1976; Triantaphyllidis et al. 1978; Bock 1980; Tsacas and Tsacas 1984 (mainly for African members); Kallantzi-Makri et al. 1985; Loukas et al. 1986; Kimura 1987; Kim et al. 1989; Lamnissou and Zouros 1989], but the phylogeny of the montium subgroup is still controversial.

Recently, interesting features have been described concerning some oriental species of the montium subgroup, features that are unique among Drosophila species. These features are the existence of well-formed Balbiani rings, a great number of unusual paired structures that may represent inverted tandem chromosomal duplications (Scouras and Kastritsis 1984; Kastritsis et al. 1986; Mavragani-Tsipidou et al. 1990a, 1990b, 1992a, 1992b; Mavragani-Tsipidou and Scouras 1991; Scouras and Mavragani-Tsipidou 1992), and an extra heat-induced puff in the salivary-gland chromosomes of strain 2 of D. auraria in this subgroup (Scouras et al. 1986). In addition, it was found that the \(\beta\)-tubulin gene family is arranged in a cluster in D. auraria, although this gene family is dispersed in D. melanogaster. The same gene cluster has been observed in D. quadraria, D. bicornuta, D. serrata, and D. jambulina; all these species are typical oriental members of the montium subgroup (Z. G. Scouras, unpublished)

1. Key words: Drosophila montium subgroup, mtDNA, restriction maps, phylogeny.

Address for correspondence and reprints: Zacharias G. Scouras, Department of Genetics, Development and Molecular Biology, School of Biology, Faculty of Sciences, Aristotle University of Thessaloniki, Thessaloniki 54006, Greece.

© 1993 by The University of Chicago. All rights reserved.
0737-4038/93/1002-0009$02.00

375
data). This suggests that the *montium* subgroup has a different genome organization and that these species are rather closely related.

In the present study we have attempted to determine phylogenetic relationships among six oriental species of the *montium* subgroup—namely, *D. auraria*, *D. quadraria*, *D. bicornuta*, *D. birchii*, *D. serrata*, and *D. jambulina*, using detailed restriction-site maps of mitochondrial DNA (mtDNA). According to Bock and Wheeler (1972), Bock (1980), and Lemeunier et al. (1986), these species occupy different geographic territories of the *D. montium* subgroup and therefore could represent the *montium* oriental geographic distribution. Among others, the results of the present study reinforce the previous idea that the above-mentioned *montium* species are rather closely related.

**Material and Methods**

**Species and Strains**

Six *Drosophila montium*–subgroup species were used in this study. The species and numbers of strains, the stock centers, and their geographic distribution (according to Lemeunier et al. 1986) were as follows:

*D. auraria*: two strains (17 and 2) isolated from an original stock, number 3040.11b, from the collection of the University of Texas (UT) (Japan).

*D. quadraria*: one stock, number 14028-0691.0 [from the Bowling Green (BG) *Drosophila* stock center] (Taiwan).

*D. serrata*: three stocks, numbers 3018.1, 3019.7, and 3022.1 (UT) (Australia).

*D. birchii*: one stock, number 14028-0521.0 (BG) (New Guinea).

*D. bicornuta*: one stock, number 3146.9 (UT) (Sumatra, Java, and Borneo).

*D. jambulina*: two stocks, numbers 3116.11 and 3120.5 (UT) (India).

Details of the specific traits and maintenance for all these strains can be found in the studies by Lemeunier et al. 1986 (for a review), Scouras and Kastritsis (1984), and Mavragani-Tsipidou et al. (1990a, 1992a, 1992b).

**mtDNA Preparation and Mapping Procedures**

mtDNA isolation from adult flies, restriction-enzyme digestions, electrophoresis, and recording of fragment-length patterns have been described elsewhere (Pissios and Scouras 1992). Twelve restriction enzymes were used; 2 (*HaeIII* and *HpaII*) recognize 4-bp sequences, and 10 (*BglII*, *ClaI*, *EcoRI*, *EcoRV*, *HindIII*, *HpaI*, *PstI*, *PvuI*, *XbaI*, and *XhoI*) recognize 6-bp sequences.

Restriction-site maps were constructed by using complete, partial, and double digestions, and tests of fragment homology among species were performed by filter hybridization (according to the method of Church and Gilbert 1984) by using known fragments of *D. melanogaster* mtDNA as probes (Pissios and Scouras 1992). These probes were obtained from *HaeIII* digests of *D. melanogaster* mtDNA separated on low-melting-temperature agarose gels and were radiolabeled with $\alpha$-P$^{32}$-dCTP, according to the random priming procedure of Feinberg and Vogelstein (1983).

**Results**

**mtDNA Restriction-Site Maps**

A total of 50 restriction sites have been detected among all restriction enzymes and strains used (fig. 1). These correspond to a sample of 1.67% of the mitochondrial genome. *PvuI* did not digest any of the mtDNAs of the strains studied. *XhoI* digested
Mitochondrial DNA Evolution in D. montium Subgroup 377

EcoRI

Mitochondrial DNA Evolution in D. montium Subgroup 377

Fig. 1 — Restriction-site alignment of the seven types of mtDNA detected in the Drosophila montium—species subgroup. The equivalent genome organization, at the bottom, is taken from Clary and Wolstenholme (1985). Numbers indicate fragment size (in bp). Gene organization is shown at lower right.

Only the mtDNAs of the sibling species Drosophila auraria and D. quadraria and that of strain 3018.1 of D. serrata, while PstI digested exclusively the mtDNAs of the D. serrata—species complex: all three strains of D. serrata and D. birchii. All other restriction enzymes tested digested the mtDNA at one to eight sites (fig. 1). Fifteen (30%) of the restriction sites are identical in all strains.

The restriction-site maps of the montium species that we have studied are presented in figure 1. The mtDNA restriction map of strain 3018.1 of D. serrata has been taken from our earlier work (Pissios and Scouras 1992), with the exception of the HpaII
digest that is presented here. Seven different types of mtDNA were established—namely, D. auraria, D. quadraria, D. serrata (two types, that of strains 3019.7 and 3022.1 being the first type and that of strain 3018.1 being the second type), D. birchii, D. bicornuta, and D. jambulina (both strain 3116.11 and strain 3120.5, one type) (see fig. 1). For comparison purposes the mtDNA were aligned at the 900-bp EcoRI fragment that is common to all Drosophila species’ mtDNA so far studied (Solignac et al. 1986; Afonso et al. 1990; Gonzales et al. 1990; present study). These maps have also been aligned with the complete mtDNA map of D. yakuba (Clary and Wolstenholme 1985). The mitochondrial genome organization can also be seen in figure 1. Although only a few strains were studied from each species, intraspecific polymorphism has been observed in the case of D. serrata (fig. 1). These data also indicate that the overall length of mtDNA among the montium species varies from 16.7 to 17.1 kb.

Nucleotide Divergence and Phylogenetic Trees

The degree of nucleotide divergence [$\delta$; number of nucleotide substitutions per site (Nei and Tajima 1983)] for every pair of strains was calculated using a computer program provided by M. Nei. In table 1 are shown the $\delta \pm SE$ (standard error) values for pairs of strains. On the basis of these $\delta$ values, two phylogenetic trees relating the seven types of mtDNA (fig. 1) of the montium subgroup were constructed (fig. 2). The tree produced by using the neighbor-joining method (Saitou and Nei 1987) allows varying rates of evolution (fig. 2) in the different lineages. Although the latter method produces an unrooted tree, a root was placed at the midpoint of the longest patristic interhaplotype distance (Farris 1972) [in our case, between D. serrata (3018.1) and D. birchii], in order to facilitate the comparisons of the relationships among species in both trees.

Our data were further evaluated, on the basis of the presence or absence of the restriction sites, by using the mix program of the phylogenetic package PHYLIP, version 3.4. According to this parsimony analysis (data not shown), the minimum number of substitutions needed to construct a tree including all the montium strains studied is 41. By this evaluation, three equivalent trees were obtained. These trees are closely related to that produced by the neighbor-joining method (fig. 2), differing only in the relative position that D. jambulina has with regard to the three clusters—namely, the two strains of D. serrata (3018.1 and 3019.7), the D. auraria–D. quadraria, and the D. birchii–D. bicornuta. Application of the bootstrap method (PHYLIP, version 3.4; Felsenstein 1985) confirmed the uncertain position of D. jambulina in the tree.

<table>
<thead>
<tr>
<th></th>
<th>D. serrata (3018.1)</th>
<th>D. serrata (3019.7)</th>
<th>D. jambulina</th>
<th>D. auraria</th>
<th>D. quadraria</th>
<th>D. birchii</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. serrata 3018.1</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>D. serrata 3019.7</td>
<td>4.26 ± 1.45</td>
<td>6.07 ± 1.88</td>
<td>6.57 ± 1.88</td>
<td>6.90 ± 1.88</td>
<td>7.52 ± 2.08</td>
<td>...</td>
</tr>
<tr>
<td>D. jambulina</td>
<td>6.07 ± 1.88</td>
<td>3.71 ± 1.32</td>
<td>5.93 ± 1.93</td>
<td>6.26 ± 1.94</td>
<td>6.79 ± 2.08</td>
<td>...</td>
</tr>
<tr>
<td>D. auraria</td>
<td>6.57 ± 1.88</td>
<td>5.93 ± 1.93</td>
<td>4.10 ± 1.41</td>
<td>4.43 ± 1.41</td>
<td>4.78 ± 1.83</td>
<td>...</td>
</tr>
<tr>
<td>D. quadraria</td>
<td>6.90 ± 1.88</td>
<td>6.26 ± 1.94</td>
<td>0.33 ± 0.33</td>
<td>6.26 ± 1.77</td>
<td>5.25 ± 1.77</td>
<td>...</td>
</tr>
<tr>
<td>D. birchii</td>
<td>7.58 ± 2.08</td>
<td>6.85 ± 2.08</td>
<td>5.78 ± 1.83</td>
<td>6.61 ± 1.83</td>
<td>5.59 ± 1.83</td>
<td>...</td>
</tr>
<tr>
<td>D. bicornuta</td>
<td>7.52 ± 2.08</td>
<td>6.79 ± 2.08</td>
<td>4.78 ± 1.83</td>
<td>5.59 ± 1.83</td>
<td>4.13 ± 1.43</td>
<td>...</td>
</tr>
</tbody>
</table>

NOTE.—$\delta$ is measured as the number of nucleotide substitutions per nucleotide site.
Mitochondrial DNA Evolution in D. montium Subgroup

1.33

2.75

D. serrata 3018.1

D. serrata 3019.7

D. jambulina

1.51

1.43

1.0

i 0.

serfufa 3078 7

1.51

serfufu 3U

79.7:

1.43

G? f bmbuhhu

D. uffrc7tk?

2.36

1

0.33

0.38

0.

quu&cviu

2.33

1.35

I

FIG. 2.—Phylogenetic relationships among the seven different nucleomorphs representing the six Drosophila montium-subgroup species, based on the δ values given in table 1 and on neighbor-joining method, with branches drawn to scale.

The latter method calculates how many times each branch appears in a standard position, among a number of equivalent trees that are produced by the algorithm of the method. The tree obtained (the majority-rule consensus tree) gives an indication concerning the confidence of the standard branches. The tree produced by the UPGMA method needs three more substitutions than does the most parsimonious trees. However, this difference is not statistically significant.

Discussion
Phylogenetic Relationships among the Montium Species

Of the species studied, Drosophila auraria and D. quadraria are considered sibling species (belonging to the D. auraria species complex), as are D. serrata and D. birchii (belonging to the D. serrata species complex). Drosophila bicornuta has a geographic distribution intermediate between those of the above-mentioned species complexes, and D. jambulina occupies mainly India (for a review concerning all the above species, see Lemeunier et al. 1986).

The phylogenetic trees in figure 2 clearly show that the sibling species D. auraria and D. quadraria are closely related. (Our preliminary results for the mtDNA of D. triauraria, another D. auraria sibling species, indicate that this species is also closely related to these two species.) Assuming a constant rate of evolution, we can estimate evolutionary times from nucleotide divergence data, though this method gives only very rough estimates. If the equation \( t(\text{Myr}) = \delta/(2\lambda) \) (Nei 1987, p. 41) is applied, where \( \lambda \) is the rate of nucleotide substitution per site per year per evolutionary lineage, and if \( 2\lambda = k \), which takes into consideration the rate for two evolutionary lineages \([k = 1.7\% /\text{Myr} \text{ (Caccone et al. 1988)}] \), then the above-mentioned two species have split off from a common ancestor rather recently, 0.2 Mya. Thus, the mtDNA data of the present study confirm the close relationships of the D. auraria species complex that were suggested by morphological, genetic, cytogenetic, protein, allozyme, and nuclear-DNA data (Bock and Wheeler 1972; Triantaphyllidis et al. 1978; Bock 1980; Scouras and Kastritis 1984; Kalantzzi-Makri et al. 1985; Kastritis et al. 1986; Loukas et al. 1986; Kimura 1987; Kim 1988; Kim et al. 1989; Lamnissou and Zouros 1989; Mavragani-Tsipidou et al. 1992a).
Although mtDNA and nuclear DNA generally seem to have similar evolutionary histories in the *D. auraria* species complex, the *D. serrata* species complex does not. Previous studies have shown that the polytene chromosomes of the three strains of *D. serrata* are indistinguishable from each other (Mavragani-Tsipidou et al. 1990a) and that the strains easily hybridize with each other (Mavragani-Tsipidou et al. 1990a; present study), indicating considerable similarity among the strains. The mtDNAs of strains 3019.7 and 3022.1 do not seem to be different (at least with the restriction enzymes used, and both are considered here as one haplotype, 3019.7), while that of the third strain (3018.1) differs significantly from them (fig. 1 and table 1). According to the equation given above, divergence between the two different strains occurred 2.5 Mya. This intraspecific polymorphism among the above-mentioned strains of *D. serrata* surpasses differences among species (table 1). Table 1 also shows that the nucleotide divergence between *D. serrata* and *D. birchii* is the highest among all *montium* species studied (if it is assumed that the divergence time between the two considered sibling species is ~4.3 Myr). A possible explanation for these discrepancies might be that the *D. serrata* strains and *D. birchii* have originated from a population highly polymorphic for mtDNA (for a review, see Monnerot et al. 1990).

The present data (fig. 2; also see Results) indicate that *D. birchii* and *D. bicornuta* are relatively distantly related to each other (divergence time 2.42 Mya). However, the mtDNA divergence between them is considerably smaller than that between *D. birchii* and *D. serrata*. At present, it is unclear how accurately the mtDNA divergence reflects the real evolutionary history of species (for a thorough discussion, see Monnerot et al. 1990).

Size Differences of mtDNA

The size difference of the mtDNAs of the *montium* oriental species studied here is in a range of 400 bp, and it is rather small in comparison with that reported for other *Drosophila* groups, where the difference is 2,000–3,000 bp (e.g., see Solignac et al. 1986; Gonzales et al. 1990). In agreement with the results reported by other investigators, variation in the mtDNA size is restricted to the A+T-rich region and is due to the different copy number of the small tandem repeats of this region (Fauron and Wolstenholme 1976, 1980). At least for the *melanogaster* and the *obscura* species subgroups, the mitochondrial genome size variation is in agreement with phylogenies based on cleavage maps of mtDNA; closely related species have mtDNAs of similar size, whereas distantly related species often show a large difference in size (Solignac et al. 1986; Gonzales et al. 1990). Therefore, the similar size of the mtDNA of the *montium* oriental species studied may be another indication that these *montium* species are closely related.

Acknowledgments

This work was supported by a grant from the Stiftung-Volkswagenwerk. We thank Drs. M. Yiangou, J. Karakoussis, M. Arsenakis, and G. Thomopoulos for helpful discussions. We gratefully acknowledge Professor M. Nei for his valuable suggestions and for the Δ-values computer program.

LITERATURE CITED


MASATOSHI NEI, reviewing editor

Received April 10, 1992; revision received June 11, 1992

Accepted June 18, 1992