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Evolutionary genetics of the *Drosophila melanogaster* subgroup

I. Phylogenetic relationships based on matings, hybrids and proteins

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ABSTRACT

Eight species belonging to the *Drosophila melanogaster* subgroup were examined genetically and biochemically for the construction of phylogenetic trees. Based on the hypothesis advanced by Watanabe and Kawanishi (1979, 1981), we have estimated the ages for the eight *Drosophila* species in the descending order to be as follows: *D. melanogaster*, *D. orena*, *D. simulans*, *D. yakuba*, *D. erecta*, *D. teissieri*, *D. mauritiana*, and *D. sechellia*. The plausible immediate ancestor for *D. orena*, *D. simulans* and *D. sechellia* appears to be *D. melanogaster* and that for *D. yakuba*, *D. teissieri* and *D. mauritiana* is *D. simulans*. On the other hand, a two-dimensional electrophoretic analysis has suggested that the subgroup consists of two complexes; they are *D. melanogaster* complex (*D. melanogaster*, *D. simulans*, *D. mauritiana*, *D. sechellia*) and *D. yakuba* complex (*D. yakuba*-*D. teissieri* pair and *D. erecta*-*D. orena* pair). Hybridization tests among the eight species have revealed that the most closely related pairs of species are the *D. simulans*-*D. mauritiana* within the complexes and the *D. mauritiana*-*D. teissieri* between the complexes.

1. INTRODUCTION

Lemeunier *et al.* (1986) summarized the general features of eight species belonging to the *D. melanogaster* subgroup. They gave precise descriptions on taxonomy, chromosomes, hybridization and ecology. Two species (*D. melanogaster* and *D. simulans*) are cosmopolitan, while the other six (*D. mauritiana*, *D. sechellia*, *D. yakuba*, *D. teissieri*, *D. erecta* and *D. orena*) are endemic to the Afrotropical region. The former four species are grouped as the *D. melanogaster* complex being closely related to each other and the latter four as the *D. yakuba* complex being rather distantly related.

Recently, many studies on the *D. melanogaster* complex have been published from the view point of evolutionary genetics. Lachaise *et al.* (1986) found from a study of hybridization tests that the most extreme isolation lies between *D. melanogaster* and its three siblings, *D. simulans*, *D. mauritiana*, *D. sechellia*. They concluded that *D. melanogaster* diverged from a common stem

first, followed by *D. sechellia*, and more recently *D. simulans* and *D. mauritiana*. Coyne and Kreitman (1986) compared the *Adh* locus of the members of the complex and proposed a very similar evolutionary tree as the above. Solignac and Monnerot (1986), from an analysis of mitochondrial (mt) DNA, speculated that *D. mauritiana* and *D. sechellia* had been derived from two different colonies of *D. simulans* in the Mauritius and Seychelles islands. Based on the chromosome banding pattern, phylogenetic trees of the *D. melanogaster* subgroup were first reported using six species by Lemeunier and Ashburner (1976). A revised version with eight species was presented later by Lemeunier *et al.* (1986). Eisses *et al.* (1979), using 18 enzyme loci, estimated a genetic distance from the electrophoretic data and constructed a similar phylogenetic tree. Ohnishi *et al.* (1983), using the two-dimensional electrophoresis for proteins, obtained another tree confirming the above two.

An attempt to construct a phylogenetic tree from the data of premating isolation among species has been proposed by Watanabe and Kawanishi (1981). They compared the degree of interspecies mating successes reciprocally and postulated two components responsible for species evolution. One is the relative age of a species since its speciation and the other is the immediate ancestor of each species. This method appears to be valid in the case of the evolutionary process of the *D. virilis* group which had been already studied chromosomally by Stone (1962) and electrophoretically by Hubby and Throckmorton (1965) and Nei (1971).

The present study was undertaken primarily to obtain a phylogenetic tree of the *D. melanogaster* subgroup using the interspecies mating data and the Watanabe—Kawanishi hypothesis. Hybridization tests were also made among the eight species to compare the post-mating and the pre-mating isolations. Finally, a phylogenetic tree using the two-dimensional electrophoresis data of proteins was constructed.

2. MATERIALS AND METHODS

Flies

The following ten strains of eight species were used.

- (1) Oregon-R strain of *D. melanogaster*.
- (2) Oita (Japan) strain of *D. simulans* collected in 1974.
- (3) Alexandria (UAR) strain *D. simulans* collected by Dr. O. Kitagawa in 1971.
- (4) Riviere Noire (Mauritius) strain of *D. mauritiana* collected in 1973 and supplied by Dr. J. David.
- (5) *D. sechellia* supplied by Dr. D. L. Hartl.
- (6) Mombasa (Kenya) strain of *D. yakuba* collected by Dr. O. Kitagawa in 1971.

- (7) Kounden (W. Cameroon) strain of *D. yakuba* collected in 1967 and supplied by Dr. L. Tsacas.
- (8) Mt. Selinda (Rhodesia) strain of *D. teissieri* collected in 1970 and supplied by Dr. L. Tsacas.
- (9) Lamto (Ivory Coast) strain of *D. erecta* collected in 1971 and supplied by Dr. L. Tsacas.
- (10) Bafut Nguemba (Cameroon) strain of *D. oreana* supplied by Dr. R. C. Woodruff.

Mating

Mating experiments were carried out using 'no-choice' method by Spieth (1968). Virgin females and males were collected from each strain. Both sexes were separately aged for two days, and ten females and ten males were put together in a vial (3×10 cm) containing standard medium. After two days of mating under a constant light, all females were dissected to determine whether or not they had been inseminated. The experiment was replicated from five (50 females) to forty (400 females) times. When we had two strains for each species such as *D. simulans* and *D. yakuba*, the same number of replicates was made for each cross.

Hybrid

The interspecific crosses were made by five one-day-old females and seven four-day-old males. They were transferred into new vials every three days and ten simultaneous replications were made for each cross. When we obtained the hybrid flies, single hybrid female or male was backcrossed to two parental males or females to check if they produce larvae or not.

Electrophoresis

The two-dimensional electrophoresis procedure used in the present study is the same as that described in Ohnishi *et al.* (1983) following O'Farrell (1975). Eight three-day-old males were sampled from each species to analyze abundant proteins of adult flies. The samples were homogenized and centrifuged, and then about 75 microliters of the supernatant were loaded onto an isoelectric focusing (IEF) gel. The IEF was carried out in 12 cm long tubes with internal diameter of 2 mm, using the same mixture of pH 3.5–10 and pH 5–8 ampholytes for a total of 6000V-hrs after prerunning for one hour. Sodium-dodecylsulfate (SDS) slab gel electrophoresis in the second dimension was carried out in 10% acrylamide for 4 hours at 200V. After running, all slab gels were fixed in 45% methanol and 10% acetic acid for one hour and stained in the same solution containing 0.02% Coomassie brilliant blue R 250 for 3 hours. They were destained in a mixture of 25% methanol and 7% acetic acid.

About 100 protein spots were detected in each SDS slab gel. They were

compared for each pair of species and counted a charge-change difference of protein spots as well as a protein spot which is found in one of given species but not in the other. Genetic distance was calculated applying Aquadro and Avise (1981)'s equation: $D=1-F=1-2N_{xy}/(N_x+N_y)$, in which N_{xy} is the number of shared loci in both species, and N_x or N_y is the total number of scored loci in each species. A phylogenetic tree based on the genetic distance was constructed according to the unweighted pair clustering method (UPG) by Nei (1975).

3. RESULTS

Phylogeny by mating preferences

The percentages of successful matings among the eight species of the *D. melanogaster* subgroup are shown in Table 1. It ranged from 82% to 99% within species crosses, suggesting that the mating period of 2 days was appropriate for this subgroup. Interspecific crosses among the eight species exhibited a great variations in the frequency of successful matings. *D. melanogaster* females mated relatively well with *D. sechellia*, *D. simulans* and *D. mauritiana* males, but did hardly with males of other species. *D. simulans* females were rather indiscriminate since they mated with males of every species except *D. orena*. *D. sechellia* females did not mate with other species males except *D. melanogaster*. On the other hand, *D. mauritiana* males were more successful in mating with the most species other than *D. sechellia*. But *D. orena* males did not mate other species at all.

The differences of successful matings in reciprocal matings for each pair or interspecific combination can be read from Table 1 and graphically presented in Fig. 1. The eight species in the figure were arranged as follows; If species A females mated more often with species B males than its reciprocal mating, the A species was put to the left of the B species. The most parsimonious order is *D. melanogaster*, *D. orena*, *D. simulans*, *D. yakuba*, *D. erecta*, *D. teissieri*, *D. mauritiana*, and *D. sechellia*. The interpretation of this order is that females of a left side species readily accept males of its right side species while females of the right species often reject males of the left species. Among the 28 pairwise combinations of species, 19 showed differences in reciprocal mating successes. These differences are presented by the 'crosses' of solid and dashed lines. A 'cross' with one heavy solid and one broken line indicates a pair showing a statistically significant difference in the reciprocal matings. Females were put above and males below. For example, *D. orena* females tended to mate more with *D. simulans* males than its reciprocal mating (*D. simulans* females \times *D. orena* males) though the difference is statistically insignificant. However, *D. simulans* females significantly succeeded in mating with *D. yakuba* males than its reciprocal mating (*D. yakuba* females

Table 1. Percent of successful matings between the eight species of the *D. melanogaster* subgroup

♀ \ ♂	Percent of successful matings							
	melanogaster	simulans	mauritiana	sechellia	teissieri	yakuba	erecta	orena
melanogaster	98 (50)	39.6 (250)	38 (100)	95.2 (105)	0 (100)	0.5 (200)	0 (100)	0 (105)
simulans	2.4 (387)	99 (200)	99 (200)	90.1 (101)	7 (200)	7.8 (400)	0.5 (200)	0 (100)
mauritiana	0 (100)	1 (200)	96 (50)	26 (100)	0 (100)	0 (200)	0 (100)	0 (102)
sechellia	4.3 (300)	0 (266)	0 (209)	98 (59)	0 (105)	0 (104)	0 (103)	0 (105)
teissieri	0 (100)	0 (200)	22.1 (371)	0.9 (105)	82 (50)	0 (200)	0 (100)	0 (104)
yakuba	0 (200)	0 (400)	2 (200)	0 (105)	2 (200)	90 (300)	1 (200)	0 (104)
erecta	0 (100)	0 (200)	3 (100)	0 (104)	4 (100)	0 (200)	92 (50)	0 (105)
orena	0 (105)	0.9 (105)	0.9 (105)	0 (105)	0 (105)	0 (104)	0 (104)	96 (55)

Numbers in parenthesis are females dissected.

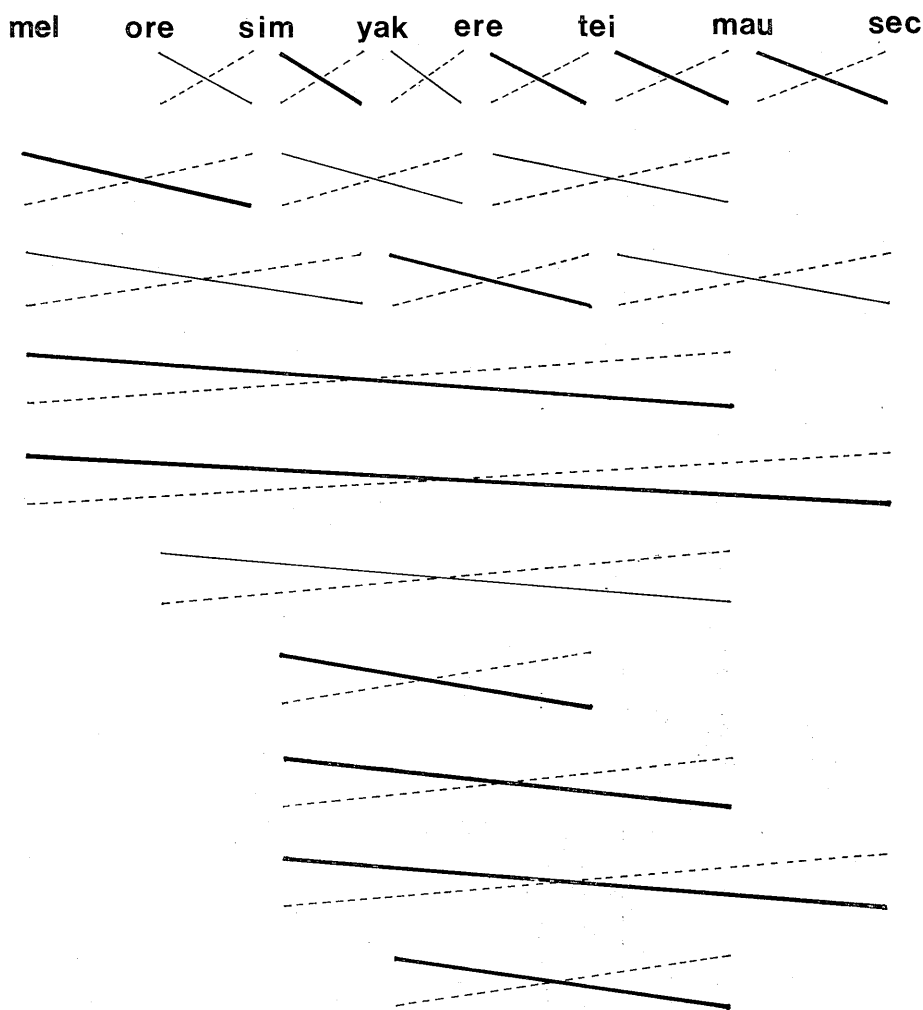


Fig. 1. Schematic relations of mating success between reciprocal crosses of the *D. melanogaster* subgroup.

× *D. simulans* males).

Watanabe and Kawanishi (1979) postulated that the order described above agrees with the evolutionary sequence, that is the birth order of species evolved. This implies therefore that *D. melanogaster* is the oldest and *D. sechellia* is the youngest among the eight sibling species. Watanabe and Kawanishi (1981) proposed a second hypothesis that the most probable ancestor of each derived species can be chosen from a group of extant species by searching the most compatible (crossable) one. For example, *D. mauritiana* was relatively younger species following *D. teissieri*. But the average crossability between them was about 11% (Table 1, $1/2(22.1\% + 0\%)$). How-

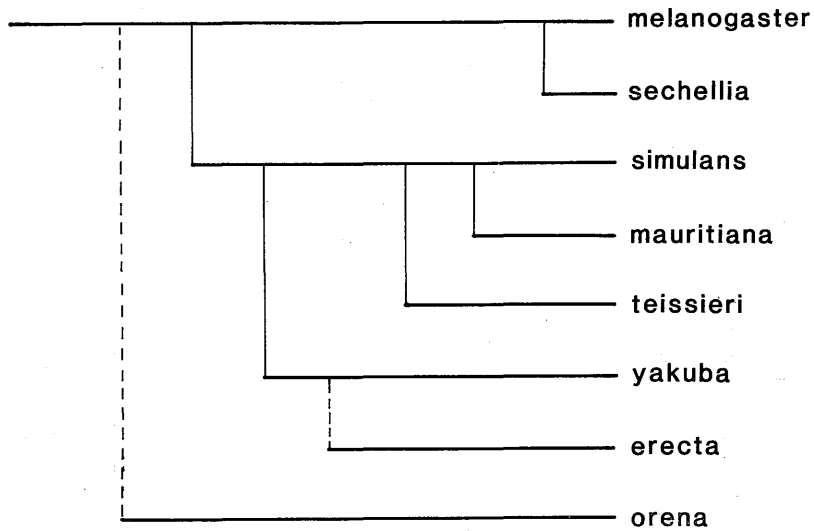


Fig. 2. Evolutionary phylogeny of the *D. melanogaster* subgroup based on the asymmetrical mating among species.

ever, the species showing the highest crossability with *D. mauritiana* was *D. simulans* ($1/2 (99\% \times 1\%) = 50\%$) rather than *D. teissieri*. Thus, the *D. mauritiana* was probably derived from *D. simulans*, but not from *D. teissieri*. Fig. 2 summarizes the phylogenetic relationship among the eight species of the *D. melanogaster* subgroup. Vertical lines present the relation between ancestors and descendants (branches), being higher possibilities in solid lines and lower possibilities in dashed lines. The length of horizontal lines presents the age of species. Therefore, *D. melanogaster* appears to be the oldest ancestor of this subgroup, firstly *D. orena* have evolved and then *D. simulans* diverged from *D. melanogaster*. From *D. simulans* three species have branched in a descending order of *D. yakuba*, *D. teissieri* and *D. mauritiana*. *D. erecta* assumed to have diverged from *D. yakuba*. *D. sechellia* diverged most recently from *D. melanogaster*.

Interspecific hybrid flies

A total of 56 different interspecific crosses were made to obtain hybrid flies. Table 2 summarizes the viability and fertility of the hybrid flies. Among the all possible crosses, 16 interspecific combinations produced hybrid flies. Some combinations, such as the cross of *D. mauritiana* females \times *D. melanogaster* males, did not mate in the previous mating experiment (Table 1) but produced hybrids in the present experiment. The discrepancy might be due to differences of mating conditions such as the age and the number of flies introduced in a vial for different mating periods.

Crosses between females of *D. melanogaster* and males of *D. simulans*, *D.*

Table 2. *Interspecific crosses between the eight species of the D. melanogaster subgroup*

♀	♂																	
		melanogaster	simulans	mauritiana	sechellia	teissieri	yakuba	erecta	orena									
melanogaster																		
simulans		♂ L ♀ S	♂ S ♀ L	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L
mauritiana		♂ L ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S
sechellia		♂ L ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S	♂ F ♀ S
teissieri		♂ 0	♂ 0	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S
yakuba		♂ 0	♂ 0	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S	♂ S ♀ S
erecta		♂ 0	♂ 0	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L	♂ S ♀ L
orena		♂ 0	♂ 0	♂ 0	♂ 0	♂ 0	♂ 0	♂ 0	♂ 0	♂ 0	♂ 0	♂ 0	♂ 0	♂ 0	♂ 0	♂ 0	♂ 0	♂ 0

F.-Fertile; S.-Sterile; L.-Lethal; O.-no F₁.

mauritiana and *D. sechellia*, produced sterile hybrid females but no males. In their reciprocal crosses, hybrid flies were all males and sterile. In the crosses among *D. simulans*, *D. mauritiana* and *D. sechellia*, hybrid flies were fertile in females, but sterile in males regardless of reciprocal matings. Crosses between males of *D. mauritiana* and females of *D. teissieri* (or *D. yakuba*) produced both sterile males and females, and the cross between *D. mauritiana* males and *D. erecta* females produced only sterile females. These results were consistent with the previous data of Lemeunier *et al.* (1986). However, as a new case, we obtained sterile hybrid females in the cross of *D. simulans* female and *D. teissieri* males.

Above results, together with the mating success shown in Table 1, suggest that (1) the four species belonging to the *D. melanogaster* complex were closely related to each other especially among the trio of *D. simulans*, *D. mauritiana* and *D. sechellia* and the four species belonging to the *D. yakuba* complex were somewhat more distantly related to each other, (2) key species combining the two complexes were females of *D. simulans* and males of *D. mauritiana*.

Phylogeny by electrophoresis data

Table 3 shows the genetic distances between species obtained by the two-

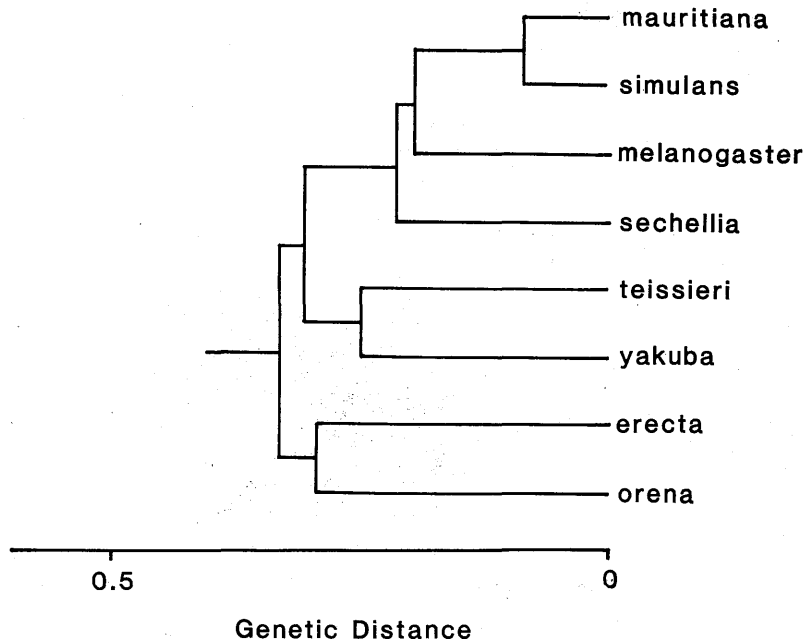


Fig. 3. A dendrogram showing the phylogenetic relationships among the eight species of the *D. melanogaster* subgroup, based on genetic distances obtained by the two-dimensional electrophoresis.

Table 3. Genetic distances among the eight species of the *D. melanogaster* subgroup, based on data from about 100 proteins obtained by two-dimensional electrophoresis

$\frac{\sigma}{\rho}$	melanogaster	simulans	mauritiana	sechellia	teissieri	yakuba	erecta	orena
melanogaster								
simulans	0.176							
mauritiana	0.214	0.085						
sechellia	0.198	0.202	0.241					
teissieri	0.262	0.256	0.235	0.301				
yakuba	0.333	0.329	0.346	0.373	0.247			
erecta	0.314	0.345	0.276	0.329	0.313	0.362		
orena	0.290	0.382	0.288	0.365	0.325	0.350	0.293	

dimensional electrophoresis, and Fig. 3 is a dendrogram constructed by the distance data. The four species of the *D. melanogaster* complex were closely related and the *D. simulans*-*D. mauritiana* pair was the closest. *D. sechellia* stands at a slightly distant place from them. Among the *D. yakuba* complex, the *D. teissieri*-*D. yakuba* pair was relatively closer to the *D. melanogaster* complex. Two other species, *D. erecta* and *D. orena*, are far distantly related from the others.

4. DISCUSSION

Two phylogenetic trees of the *Drosophila melanogaster* subgroup were constructed from genetical and biochemical data. They turned out to be basically similar to each other and mostly consistent with the previous results by the chromosomal, genetical, biochemical and molecular studies (Lemeunier *et al.* 1986; Lachaise *et al.* 1986; Eisses *et al.* 1979; Ohnishi *et al.* 1983; Coyne and Kreitman 1986; Solignac and Monnerot 1986). Eight species of the *D. melanogaster* subgroup can be divided into two complexes. The *D. melanogaster* complex consists of *D. melanogaster*, *D. simulans*, *D. mauritiana* and *D. sechellia*, and the *D. yakuba* complex consists of *D. yakuba*, *D. teissieri*, *D. erecta*, *D. orena*. The members of the former complex are relatively closer to each other, while those of the latter complex are more distantly related. *D. simulans* and *D. mauritiana* are the most closely related pair in the subgroup.

The present paper emphasized the usefulness of the method which constructs a phylogeny from data of the mating preference experiment. Comparing it with the results obtained by other methods, it will give a further insight into understanding of evolutionary process of species.

The origin of D. mauritiana: Since the discovery of *D. mauritiana* (Tsacas and David 1974) it has been studied chromosomally (Lemeunier and Ashburner 1976), genetically (David *et al.* 1976) and biochemically (Eisses *et al.* 1979; Gonzalez *et al.* 1982). These results conclude that *D. simulans* and *D. mauritiana* are much closer than they are to *D. melanogaster*. In the light of the hypothesis that females of the ancestral species and males of the derived species are more compatible than vice versa, Watanabe and Kawanishi (1979) proposed that the above three species had evolved in the order of *D. melanogaster*, *D. simulans* and *D. mauritiana*. David *et al.* (1974) had previously proposed that *D. mauritiana* is a species derived from geographic subpopulation of *D. simulans*. Robertson (1983) analysed mating behavior of the three species and concluded that *D. mauritiana* has diverged from *D. simulans* in a number of ways. Solignac and Monnerot (1986) compared mt DNA and speculated that the first invasion of *D. simulans* into Mauritius led to *D. mauritiana* and the second invasion of the same species carried the other

type of mt DNA into some *D. mauritiana* cytoplasm. Thus, the ancestral species of *D. mauritiana* so far studied to be *D. simulans*.

The origin of D. sechellia: Unlike *D. mauritiana*, the evolutionary status of *D. sechellia* (Tsacas and Bachli 1981) is rather nebulous. This species is morphologically intermediate between *D. simulans* and *D. mauritiana* and chromosomally homosequential to them (Lemeunier and Ashburner 1984), and it hybridizes with the both species, producing fertile females and sterile males. In addition, these three species behaved similarly when crossed to *D. melanogaster*, producing unisexual sterile hybrids (Lemeunier *et al.* 1986 and the present data). These results appear to imply that the trio of *D. simulans*, *D. mauritiana* and *D. sechellia* are closely related to each other and they are slightly distant from *D. melanogaster*.

Coyne and Kreitman (1986) compared the DNA sequence of the *Adh* locus and confirmed the above conclusion. They further asserted that the *simulans/sechellia* divergence occurred more recently than the *simulans/mauritiana* divergence. On the other hand, from the hybridization tests, Lachaise *et al.* (1986) found that *D. sechellia* was slightly more isolated among the trio and concluded that *D. melanogaster* diverged first, followed by *D. sechellia* and, most recently, *D. simulans* and *D. mauritiana*. The unsolved problem is estimations of the relative ages of *D. sechellia* and its ancestor.

Two phylogenetic trees of our present study also showed a discrepancy in this problem. From the phylogeny based on the mating preference, *D. sechellia* seems the youngest and most recently derived from *D. melanogaster* not from *D. simulans*. On the other hand, the biochemical data predict that the *D. simulans/D. mauritiana* divergence was more recent than the *D. sechellia* from the other members of the *D. melanogaster* complex. Although we need further experiments using more species strains, we can speculate reasons explaining the above discordance. (1) Old polymorphism in populations of *D. melanogaster* or *D. simulans* before the emergence of *D. sechellia*; A strain of *D. melanogaster* carrying those genetic variation of *D. simulans* and the same mating propensity as the present *D. melanogaster* might have been a partial ancestor of *D. sechellia*. (2) Recent introgression of *D. simulans* into *D. sechellia*; Females of *D. simulans* mate easily with *D. sechellia* males producing fertile hybrid females. Contaminations by the genome of *D. simulans* might have further complicated the evolutionary history of *D. sechellia*.

The origin of D. teissieri and D. yakuba: Among the *D. yakuba* complex *D. teissieri* is the closest species to the *D. melanogaster* complex especially to *D. simulans* and *D. mauritiana*. In the phylogeny based on the mating data, three species have originated from the *D. simulans* lineage, *D. yakuba* first, *D. teissieri* second and *D. mauritiana* third. These three species are in a

relation of sistership sharing the same mother of *D. simulans*. Interspecific hybrids between *D. simulans* females and *D. teissieri* males produce only sterile females. But we have not obtained hybrids between *D. simulans* females and *D. yakuba* males. Males of *D. mauritiana* produced sterile progenies when crossed to females of *D. teissieri* and *D. yakuba*. Genetic distance data always showed that *D. teissieri* is much closer to the *D. melanogaster* complex than *D. yakuba* or the other species of the *D. yakuba* complex.

The evolutionary scenario of the D. melanogaster subgroup: Bock and Wheeler (1972) summarized the taxonomy and the distribution of the *D. melanogaster* subgroup, suggesting that both *D. melanogaster* and *D. simulans* could have evolved in the Aethiopian biogeographical zone and have only become widespread relatively recently in association with human movements. Lemeunier *et al.* (1986) stated that the eight species of the *D. melanogaster* subgroup clearly originated in the Afrotropical region, and cited the conclusion of Lachaise *et al.* (in preparation) that the *D. orena*/*D. melanogaster* divergence time was estimated to be 2–6 Myr, the pre-*melanogaster*/pre-*simulans* split as 0.8–3 Myr and the *D. simulans*/*D. mauritiana* divergence as 0.4–1 Myr. These conclusions are well consistent with the chromosome phylogeny derived by Lemeunier *et al.* (1986).

Phylogenetic studies so far reported suggest that *D. melanogaster* is an ancestral species, and that *D. mauritiana* and *D. sechellia* are rather young species. However, since we have no clear fossil record of *Drosophila* species, the evolutionary history can not accurately be estimated. We only speculate the evolutionary process using some of assumptions. Watanabe and Kawanishi (1979, 1981) proposed a hypothesis that (1) females of the ancestral species mate well with its descendant species males but not in the reciprocal combination and (2) the probable ancestor can be specified among the older species by searching a pair of species which exhibits a high degree of compatibility. In fact they applied successfully this method to the *D. virilis* group and revealed significant findings relevant to its evolution.

The estimated sequential order of these speciations and the divergence branches are shown in Fig. 2. *D. melanogaster* is the most ancestral species among the subgroup. In a very early stage, may be 2–6 Myr ago, *D. orena* and, about 0.8–3 Myr ago, *D. simulans* diverged from *D. melanogaster*. From *D. simulans*, three species, *D. yakuba*, *D. teissieri* and *D. mauritiana*, had evolved successively during the past half million years. Before the emergence of *D. teissieri*, *D. erecta* was perhaps born from *D. yakuba*. Finally, *D. sechellia* appeared from *D. melanogaster* very recently. Ancestral species might have been different from the present species. Therefore we should call, for instance, pre-*D. melanogaster* instead of *D. melanogaster* when we refer to the species at the time of divergences of *D. orena*, *D. simulans* and *D. sechel-*

lia. Moreover, these pre-*D. melanogaster* might have been different from each other. However, the mating propensities of these pre-*D. melanogaster* species have been maintained in the present *D. melanogaster* since the character is very important for the identity of species. At present, we have no conclusive evidence supporting the theory that the asymmetrical mating characteristics between ancestral and descendant species indicate the direction of species evolution. However the evolutionary scenario inscribed in the mating behavior seems to be remarkably correct, and consistent with many circumstantial evidences.

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