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Evolutionary Genetics of the *Drosophila montium* Subgroup. II. Mitochondrial DNA Variation

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ABSTRACT—Mitochondrial DNA (mtDNA) variation was investigated among 18 species in the *Drosophila montium* species subgroup. Based on the restriction patterns of 14 restriction enzymes, the subgroup was clearly classified into 3 species complexes, the *kikkawai* complex (6 species), the *jambulina* complex (4 species) and the *auraria* complex (8 species), which were previously proposed by cross experiments. The relationship between the *kikkawai* and the *jambulina* complex were proved to show much closer than that between the *auraria* and the two complexes by the proportion of restriction fragments which were shared among complexes. The phylogeny among members within the complexes constructed using mtDNA variation was not always consistent with other available information concerning about protein divergence and reproductive isolation.

INTRODUCTION

The *Drosophila montium* species subgroup is the largest subgroup in the *D. melanogaster* species group and is found throughout south-east Asia and tropical Africa. Seventy nine species have been described in this subgroup [10], and a variety of stages of speciation process, which provide useful materials for the study of evolutionary genetics, are recognized. The phylogenetic relationships between members of this subgroup have been investigated by biochemical analysis [13, 14], chromosomal analysis [2, 3], and cross experiments [8, 9].

Kim *et al.* [9] examined the crossability of 272 interspecific combinations among 17 species of this subgroup and classified the species into three groups of species, or 'species complexes', which were defined as species groups producing viable and fertile hybrids: the *kikkawai* complex (6 species), the *jambulina* complex (4 species) and the *auraria* complex (7 species). The classification is very similar to that predicted biochemically [14]. However, phylogenetic relationships within each species complex based on unsuccessful mating values [9] and 2-dimensional electrophoresis (2DE) [14] did not always coincide with each other.

Restriction analysis of mitochondrial DNA (mtDNA) has been a useful tool for estimating genetic diversity among populations and closely related species [1, 4, 5, 16, 17]. The advantages of this method are remarkable when phylogenetic relationships among very closely related species are considered. In the present study, we reexamined the phylogenetic relationships of eighteen *D. montium* subgroup species using restriction pattern analysis of mtDNA and compared it to those of previously reported using cross experiments or biochemical analyses.

MATERIALS AND METHODS

Table 1 lists 18 species from the *D. montium* subgroup investigated. Except *D. lacteicornis,* the same strains that Kim *et al.* [9] examined were used. The strain of *D. lacteicornis* was the same as used by Ohnishi and Watanabe [14]. All strains used were established from single wild-caught females.

MtDNAs were prepared by the methods of Tamura and Aotsuka [18], with some modifica-

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Table 1. Species of the *D. montium* species subgroup used in this study

Species	Source
D. pennae	Texas stock no. 3028.1, Papua New Guinia
D. bocki	AO-1, Thailand
D. kikkawai	Okinawa, Japan
D. leontia	AO-2, Thailand
D. lini	Texas stock no. 3146.1, Taiwan
D. lini-like	MMY326, Maymyo, Myanmar, 1981
D. barbarae	Texas stock no. 3033.1, Malaysia
D. jambulina	TMU, India, 1979
D. panjabiensis-like	Texas stock no. 3116.1, Thailand
D. panjabiensis	TMU, India, 1979
D. quadraria	Texas stock no. 3075.1, Taiwan
D. triauraria	Tsukuba, Japan, 1976
D. auraria	Mishima, Japan, 1978
D. biauraria	B660, Hokkaido, Japan, 1978
D. subauraria	KT-4, Kitagami, Japan, 1982
D. rufa	Mishima, Japan, 1978
D. yuwanensis	Amamiohsima, Japan, 1987
D. lacteicornis	IRO78, Iriomotejima, Japan, 1978

tions. Fourteen restriction enzymes were used to reveal mtDNA variation; *Ava*I, *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I, *Sac*I, *Sty*I, *Xba*I (recognize 6 bp sequences), *Hae*III, *Hha*I, *Hinf*I, *Hpa*II, *Rsa*I (recognize 4 bp sequences). The restriction fragments were separated by electrophoresis in 1% agarose gel and visualized with ethidium bromide. The *Sty*I fragments of lambda phage DNA were used as size standards.

RESULTS

Table 2 summarizes the restriction patterns of mtDNA digested by 14 restriction enzymes in 18 species of *D. montium* species subgroup. The restriction patterns of a given enzyme observed in *D. kikkawai* were designated by "a". The remaining patterns apparently differing from "a" by a single or multiple restriction site changes were designated as "b", "c" and so on.

Among 14 restriction enzymes, *Hha*I and *Pst*I yielded the same cleavage pattern in all the species. *D. quadraria* and *D. triauraria* was the only species-pair that could not be distinguished by the restriction analysis. Taking into account all restriction patterns, the 18 species seemed to be divided into 3 groups as shown in Table 2, the *kikkawai, jambulina* and *auraria* complex. The grouping was very similar to those predicted by 2DE analysis [14] and by mating preference [9].

There were several diagnostic restriction patterns which represented each species complex. For example, two or three restriction patterns for *Rsa*I were identified in every species complex, but none were shared between the complexes. The proportion of shared restriction patterns between the *kikkawai* and *jambulina* complexes were apparently larger than between these two and the *auraria* complex. Between the *kikkawai* and *jambulina* complexes, some of digested patterns for all but one (*Rsa*I) restriction enzymes were shared. On the other hand, the *auraria* complex has many complex-specific restriction patterns. Restriction patterns for 7 (*Hpa*II, *Hae*III, *Rsa*I, *Eco*RI, *Sac*I, *Bam*HI and *Sty*I) of 14 restriction enzymes of the *auraria* complex were unique to this complex. These findings allowed us to conclude that the *kikkawai* and *jambulina* complexes were much closer to each other than the *auraria* and the two complexes.

To investigate the relationships among members within the complexes, expected substitution rates

TABLE 2. Restriction patterns of mtDNA in 18 species of the *D. montium* species subgroup

species	*Hpa*II	*Hae*III	*Hinf*I	*Hha*I	*Rsa*I	*Xba*I	*Eco*RI	*Bgl*II	*Ava*I	*Hin*dIII	*Sac*I	*Pst*I	*Bam*HI	*Sty*I
kikkawai complex														
pennae	a	c	b	a	a	a	a	a	a	a	b	a	a	a
bocki	b	d	b	a	a	a	a	a	a	b	b	a	a	a
kikkawai	a	a	a	a	a	a	a	a	a	a	a	a	a	a
leontia	c	b	c	a	a	a	a	a	a	a	b	a	a	a
lini	a	e	d	a	b	a	b	a	a	c	b	a	a	a
lini-like	a	e	b	a	b	a	a	a	a	c	b	a	a	a
jambulina complex														
barbarae	d	a	e	a	c	a	b	a	a	d	c	a	a	a
jambulina	a	f	f	a	d	a	c	b	b	d	c	a	a	a
punjabiensis-like	e	f	f	a	d	a	d	b	a	a	c	a	a	a
punjabiensis	e	f	a	a	c	a	d	b	a	d	a	a	a	a
auraria complex														
quadraria	f	g	g	a	e	a	e	b	a	d	d	a	b	b
triauraria	f	g	g	a	e	a	e	b	a	d	d	a	b	b
auraria	f	g	g	a	e	a	e	b	a	d	d	a	b	b
biauraria	f	g	b	a	e	a	e	b	a	a	d	a	b	b
subauraria	g	g	g	a	f	a	e	b	a	d	e	a	b	b
rufa	h	g	g	a	g	a	f	b	a	d	f	a	b	b
yuwanensis	h	h	b	a	g	a	e	b	a	e	f	a	b	c
lacteicornis	h	g	b	a	g	b	f	b	a	d	f	a	b	b

TABLE 3. MtDNA differentiation among species in the *kikkawai* complex

species	*pen.*	*boc.*	*kik.*	*leo.*	*lin.*	*lin-l.*
pennae	(24 18)	0.0333	0.0298	0.0314	0.0567	0.0549
bocki	15 12	(24 18)	0.0367	0.0277	0.0598	0.0574
kikkawai	15 13	13 14	(23 17)	0.0286	0.0535	0.0509
leontia	15 14	16 14	15 15	(25 18)	0.0533	0.0514
lini	11 9	11 8	12 8	12 9	(23 20)	0.0125
lini-like	11 10	11 9	12 9	12 10	20 16	(24 19)

The figures above the diagonal are expected substitution rates(d), and those below the diagonal are numbers of shared restriction fragments (left; 6-cutter enzymes, right; 4-cutter enzymes) for each pair of species. The numbers of restriction fragments for each species are on the diagonal.

TABLE 4. MtDNA differentiation among species in the *jambulina* complex

species	*bar.*	*jam.*	*pun-l.*	*pun.*
barbarae	(23 18)	0.0668	0.0512	0.0623
jambulina	9 8	(22 18)	0.0499	0.0551
punjabiensis-like	11 10	11 10	(21 22)	0.0204
punjabiensis	10 8	10 10	16 16	(22 20)

See Table 3 for other explanation.

Table 5. MtDNA differentiation among species in the *auraria* complex

species	*qua.*	*tri.*	*aur.*	*bia.*	*sub.*	*ruf.*	*yuw.*	*lac.*
quadraria	(17 21)	0.0000	0.0113	0.0321	0.0498	0.058	0.0625	0.0582
triauraria	17 21	(17 21)	0.0095	0.0321	0.0498	0.0581	0.0625	0.0582
auraria	15 17	15 18	(17 21)	0.0321	0.0498	0.0581	0.0625	0.0582
biauraria	10 16	10 16	10 16	(18 22)	0.0241	0.0545	0.0582	0.0539
subauraria	7 16	7 16	7 16	11 20	(18 21)	0.0670	0.0707	0.0625
rufa	7 10	7 10	7 10	8 10	7 8	(16 21)	0.0163	0.0072
yuwanensis	7 10	7 10	7 10	8 10	7 8	14 16	(19 20)	0.0181
lacteicornis	7 11	7 11	7 11	7 11	7 10	15 19	14 16	(18 20)

See Table 3 for other explanation.

for each pair of mtDNA (d) were estimated based on the proportion of shared restriction fragments by the two genomes (length-difference method) according to the formula of Nei and Li [12]. The matrices of data are shown in Table 3, 4 and 5. Since the total length of mtDNA was different in different species complexes, Nei and Li's formula was not applicable for estimating nucleotide divergence among species complex.

The average substitution rates ($\pm$SE) within a species complex were 0.0425 ± 0.0037 (the *kikkawai* complex), 0.0510 ± 0.0066 (the *jambulina* complex) and 0.0441 ± 0.0040 (the *auraria* complex). Based on the matrices of d values, the phylogenetic tree of members of the *D. montium* species subgroup was constructed by the UPGMA method of clustering [15] (Fig. 1). In this tree the connections of species complexes were not based on a numerically determined index because we did not estimate d among the complexes for the reason mentioned above. However, the closeness between the *kikkawai* and *jambulina* complexes was apparent. Therefore, we made a cluster including these two and then connected it to the cluster of the *auraria* complex.

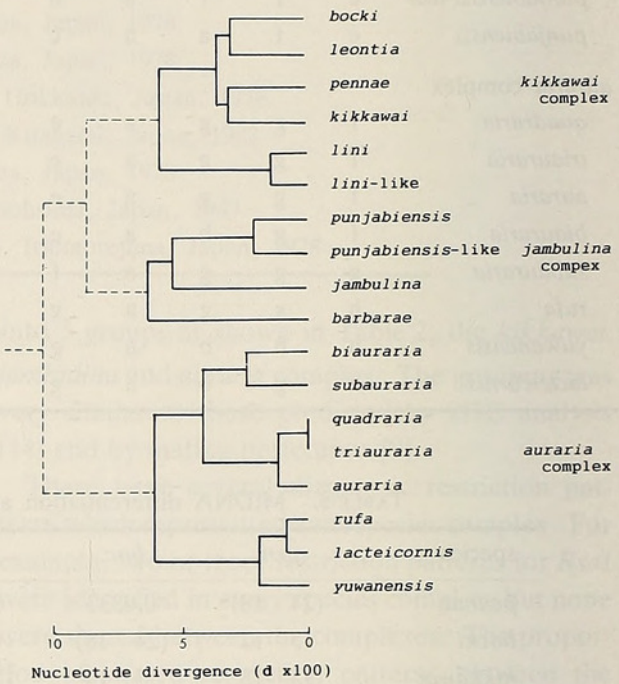

Fig. 1. Dendrogram of the 18 species of the *D. montium* species subgroup obtained by UPGMA method, based on the matrices of d(Table 3–5). The connections of species complex (dashed line) are arbitrary (see text).

The branching orders within the species complex shown in Fig. 1 are not always compatible with those predicted by protein analysis or by cross experiment. In the *kikkawai* complex, the d value between *D. lini* and *D. lini-like* was the smallest, so that they were clustered first. These species, however, were rather distantly related judging from protein differentiation, and premating isolation between them was almost complete. MtDNA genotypes of *D. barbarae* and *D. jambulina* in the *jambulina* complex were mostly different and only a few crosses were successful between these species. But *D. barbarae* and *D. jambulina* were found to be the closest species-pair in the complex by protein analysis. The topology of the tree for the *auraria* complex was very similar to those by cross experiment or 2DE-electrophoretic analysis except for the position of *D. lacteicornis*. *D. lacteicornis* shared many restriction patterns with *D. rufa* and *D. yuwanensis* which are members of

the *auraria* complex (Table 2). Some crosses among these three species were successful and yielded fertile female progeny (Kim unpublished data). However, protein configuration of *D. lacteicornis* on the 2DE-electrophoresis was significantly different from those of other species.

DISCUSSION

In the present investigation, eighteen species of the *D. montium* species subgroup were divided unambiguously into 3 groups based on mtDNA fragment patterns. This result is coincident with the subdivision of species into 3 species complexes, the *kikkawai* complex, the *jambulina* complex and the *auraria* complex, proposed by Kim *et al.* [9], as well as with the biochemical classification by 2DE [14].

Since the complexes were almost completely isolated by reproductive barriers [9], the phylogenetic relationships between complexes could not be examined by means of cross experiment. The comparison of mtDNA restriction patterns (Table 2) enabled us to conclude that the *kikkawai* and *jambulina* complexes were the closest among the complexes. Although a similar relationship among the species complexes was suggested by 2DE analysis [14], mtDNA restriction analysis seems to be more useful for grouping of closely related species since restriction patterns offer us multiple diagnostic characters.

The relationship among members within a complex varied in different measures of genetic differentiation. Kim *et al.* [9] examined the correlation between the genetic distance (D) by 2DE electrophoresis [14] and the frequency of unsuccessful matings (UM) obtained by their cross experiments. None of the coefficients of correlation within complexes were statistically significant. We estimated the coefficients of correlation between mtDNA substitution rate (d) and the other two measurements (Table 6). Among six combinations of data, only one case, between d and D in the *auraria* complex, showed a significant positive correlation, and no significant correlation was observed between premating isolation (UM) and protein or mtDNA diversities (d and D).

Coyne and Orr [7] investigated the correlation between pre- and postzygotic isolation and allozyme diversity estimated by Nei's genetic distance [11] from literature data on 119 pairs of *Drosophila* species, and found that both forms of isolation were significantly correlated with Nei's genetic distance. Assuming the constancy of allozyme diversity over time, they concluded that reproductive barriers develop gradually with time. At present it is not clear why we failed to get a significant correlation between sexual isolation and molecular diversity in the *D. montium* species subgroup. It must be noted, however, that our investigation focused on the very early stages of speciation in *Drosophila.* Coyne and Orr [7] included many species-pairs in which reproductive isolation was almost complete in their correlation estimates. Such relatively distant species-pairs may partially be responsible for the significance of the correlation since most genetic traits between distantly related species have considerably differentiated. In the *montium* species subgroup, the correlation between the frequency of unsuccessful matings and the protein difference was statistically significant when all combinations of species, in-

TABLE 6. Coefficient of correlation between different measurements of genetic diversities

speciec complex		D-d	d-UM	D-UM
kikkawai	complex (n=15)	0.3900	0.1645	0.2928
jambulina	complex (n=6)	0.2689	0.3218	0.2750
auraria	complex (n=15)	0.9136*	0.3418	0.4549

D: genetic distance (by 2DE)
d: nucleotide divergence (mtDNA)
UM: % of unsuccsessful mating
*: statistically significant at 0.1% level

cluding inter-complex comparisons, were considered [9]. Thus, the present and previous observations [9, 14] indicate that the development of reproductive isolation does not always accompany the accumulation of genetic divergence at the molecular (protein or mtDNA sequence) level, especially in the very early stages of the speciation process such as species complex formation.

Even though a large amount of data concerning genetic divergence among closely related species has been accumulated, we know little about how and what genetic divergence causes reproductive isolation [6]. More detailed genetic analysis of reproductive isolations among closely related species will be necessary in the future. In such work, mtDNA restriction analysis and the phylogenetic relationships constructed from it will be helpful for groupings of closely related species.

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