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Phylogenetic Relationships and Climatic Adaptations in the *Drosophila takahashii* and *montium* Species Subgroups

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We analyze phylogenetic relationships among temperate, subtropical highland, and subtropical lowland species of the *Drosophila takahashii* and *montium* species subgroups based on sequence data of COI and *Gpdh* genes and discuss the evolution of temperate species in these subgroups with reference to their climatic adaptations. In the *takahashii* subgroup, *D. lutescens* (the temperate species) branched off first in the tree based on the combined data set, but *D. prostipennis* (the subtropical highland species) branched off first in the trees based on single genes. Thus, phylogenetic relationships in this subgroup are still ambiguous. In the *montium* subgroup, the cool-temperate species are phylogenetically close to the warm-temperate species, and these cool- and warm-temperate species form a cluster with the subtropical highland species. This suggests that perhaps the cool-temperate species derived from the warm-temperate species and the warm-temperate species derived from the subtropical highland species. In comparison with the subtropical lowland species, the subtropical highland species may be better able to colonize temperate areas since, as in the temperate species, they have an ability to develop their ovaries at moderately low temperature. However, the subtropical highland species, as well as the subtropical lowland species, were much less cold tolerant than the temperate species. Therefore, considerable genetic reformation would be required for both the subtropical highland and the subtropical lowland species to adapt to temperate climates. © 2000 Academic Press

INTRODUCTION

Many taxa of insects are assumed to have originated in the tropics, and some of them have succeeded in colonizing temperate or arctic areas. The *Drosophila melanogaster* species group is believed to have originated and diversified in the Paleotropical regions (Throckmorton, 1975; Lemeunier *et al.*, 1986). This group is quite large, containing more than 160 species unevenly divided into 12 subgroups (Toda, 1991). With the exception of the species dispersed through human activities, temperate species are known only in the *montium*, *takahashii*, *suzukii*, and *ficusphila* species subgroups and they are distributed only in east Asia. The present study aims to follow the evolutionary routes of temperate species in the *takahashii* and *montium* subgroups.

Members of these two subgroups have been reported not only from temperate areas and subtropical lowlands but also from subtropical highlands (Chen *et al.*, 1988; Kimura *et al.*, 1994). Therefore, there is a possibility that tropical or subtropical lowland species had first adapted to subtropical highlands and then colonized temperate regions. To examine this possibility, we analyzed the phylogenetic relationships among temperate, subtropical highland, and subtropical lowland species belonging to these subgroups, based on sequence data of mitochondrial cytochrome oxidase subunit I (COI) and nuclear glycerol-3-phosphate dehydrogenase (*Gpdh*) genes, which have been extensively used for phylogenetic analysis in *Drosophila* (Barrio and Ayala, 1997; Gleason *et al.*, 1997; Kwiatowski *et al.*, 1997; Goto *et al.*, 1999). Phylogenetic relationships in these subgroups have been studied by a number of workers (Watanabe *et al.*, 1982; Ohnishi *et al.*, 1983; Ohnishi and Watanabe, 1984; Kim *et al.*, 1989, 1993; Shyamala and Ranganath, 1990; Inaba *et al.*, 1993; Parkash *et al.*, 1994), but information on the subtropical highland species is limited. In addition, the previous studies were based on protein differences detected by two-dimensional electrophoresis, allelic frequencies, restriction analyses, or cross experiments, but not on DNA sequences.

In addition, we compare climatic adaptations of temperate, subtropical highland, and subtropical lowland species on the basis of information obtained in the present and previous studies (Kimura, 1988; Kimura *et al.*, 1994; Ohtsu *et al.*, 1993, 1998; Goto and Kimura, 1998; Hori and Kimura, 1998) to understand what

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TABLE 1

Experimental Species, Ecotypes, Collection Localities, and Accession Numbers for COI and *Gpdh* Genes

Species group / Species subgroup / Species	Ecotype	Collection locality	Accession No.[a] COI	*Gpdh*[b]	
obscura group					
D. bifasciata Pomini	—	—	U51611	U47883	
melanogaster group					
melanogaster subgroup					
D. melanogaster Meigen	—	—	U37541	X61223	
D. simulans Sturtevant	—	—	M57909	AF085163	
D. teissieri Tsacas	—	—	U51618	U47809	
takahashii subgroup					
D. lutescens Okada	warm-temperates	Takamatsu (34°N)	AB027267	AB027276	AB027281
D. trilutea Bock and Wheeler	subtropical highlands	Taipei (25°N)	AB027261	AB027270	AB027286
D. prostipennis Lin	subtropical highlands	Taipei	AB027266	AB027275	AB027282
D. takahashii Sturtevant	subtropical lowlands	Naha (26°N)	AB027264	AB027273	AB027284
montium subgroup					
D. biauraria Bock and Wheeler	cool-temperates	Sapporo (43°N)	AB027259	AB027278	AB027279
D. triauraria Bock and Wheeler	cool-temperates	Onuma (42°N)	AB027262	AB027271	AB027287
D. rufa Kikkawa and Peng	warm-temperates	Takamatsu	AB027265	AB027274	AB027283
D. trapezifrons Okada	subtropical highlands	Taipei	AB027263	AB027272	AB027288
D. constricta Chen, Shao and Fan	subtropical highlands	Jihyüeh T'an (23°N)	AB027268	AB027277	AB027280
D. watanabei Gupta	subtropical lowlands	Kaohsuing (22°N)	AB027260	AB027269	AB027285

[a] Accession number of the sequence in DDBJ/GenBank/EMBL.
[b] Two accession numbers of *Gpdh* indicate the exons 3 and 4, respectively.

traits have changed along with colonization in subtropical highlands and temperate areas.

MATERIALS AND METHODS

Flies

The experimental species, their ecotypes, and collection localities are given in Table 1. Their ecotypes (cool-temperates, warm-temperates, subtropical highlands, and subtropical lowlands) have been defined according to their distributions, temperature tolerances, and diapause traits reported in previous studies (Kimura, 1984, 1988; Lemeunier *et al.*, 1986; Kimura *et al.*, 1994; Ohtsu *et al.*, 1993; Goto and Kimura, 1998; Hori and Kimura, 1998) and according to our preliminary collection in early September, 1992, in Taiwan (Table 2).

TABLE 2

Individual Numbers of *D. trapezifrons*, *D. constricta*, and *D. watanabei* Collected at Different Altitudes in Taiwan

Species	Altitude (m) 1600	1100	200
D. trapezifrons	54	7	—
D. constricta	—	5	—
D. watanabei	—	—	7

After collection, the experimental stocks were maintained for a few months to several years in the laboratory before experiments. Cornmeal–malt medium was used for rearing.

DNA Extraction

To perform PCR (polymerase chain reaction), DNA was extracted from the species studied according to the method of Goto *et al.* (1998). RNAs in the samples were digested with RNase A.

Sequencing of COI

The primers used to amplify the COI fragment were F- and R-COI (Gleason *et al.*, 1997; Goto *et al.*, 1999) (Table 3). PCR used 100 ng of DNA, 1 U of AmpliTaq

TABLE 3

Primers Used for the Amplification and Sequencing of COI and *Gpdh*

Primer	Sequence (5′-3′)
For COI	
F-COI	CCA GCT GGA GGA GGA GAT CC
R-COI	CCA GTA AAT AAT GGG TAT CAG TG
For *Gpdh*	
GNL-mel	GTG GTG CCC CAC CAG TTC AT
GNR-mel	GGC TTG AGC TGA TTT GTG CA
L4BN	CCA TGY GCT GTC TTG ATG GG
R4M	ACA GCC GCC TTG GTG TTG TCG CC
Gpdh-F	TCA AGC TCG GCG ACA ACA
Gpdh-R	CCC ATC AAC ACG GCG CAT GG

DNA polymerase (Perkin–Elmer), and a final concentration of 1.5 mM of $MgCl_2$, 1× PCR buffer II as formulated by Perkin–Elmer, 0.4 μM of F- and R-COI primers, and 0.2 mM of dNTP in a total volume of 50 μl. Amplification was performed with 35 cycles of 30 s denaturing at 94°C, 30 s annealing at 50°C, and 30 s extension at 72°C. The length of amplified products agreed with that of the COI reported by Gleason *et al.* (1997).

The amplified fragments were purified using QIAquick Gel Extraction Kit (QIAGEN). The sequences were obtained from an ABI 373A automated sequencer (PE Applied Biosystems) with a DNA sequencing kit (Dye Terminator Cycle Sequencing Ready Reaction; PE Applied Biosystems) according to suppliers' instructions.

Cycle sequencing was performed using F-COI and R-COI primers. The accession numbers for COI in DDBJ/GenBank/EMBL are given in Table 1.

Sequencing of Gpdh

The primers used to amplify the *Gpdh* fragment were GNL-mel and GNR-mel, designed on the basis of the *Gpdh* sequence derived from *D. melanogaster* Meigen (the GenBank Accession No. is X61223) (Table 3). The first bases correspond to positions 3351 and 4543 in the *D. melanogaster* sequence, respectively. PCR components were the same as for the amplification of COI, except for the primers. Amplification was performed with 35 cycles of 30 s denaturing at 94°C, 30 s annealing at 57°C, and 90 s extension at 72°C. The amplified fragments were purified as described above.

For cycle sequencing of the *Gpdh* fragments, we designed two more primers, Gpdh-F and Gpdh-R, on the basis of the sequence derived from *D. melanogaster* (the GenBank Accession No. is X61223) (Table 3). The first bases correspond to positions 3795 and 3555 in the *D. melanogaster* sequence, respectively.

Cycle sequencing was performed using GNL-mel, L4BN, R4M, Gpdh-F, and Gpdh-R primers (Barrio and Ayala, 1997; Goto *et al.*, 1999) (Table 3). The Accession Nos. for *Gpdh* in DDBJ/GenBank/EMBL are given in Table 1.

Phylogenetic Inference

As an outgroup, *D. bifasciata* (the *obscura* species group) was used. In addition, three species of the *melanogaster* species subgroup (*D. melanogaster, D. simulans,* and *D. teissieri*) were added to the analysis to determine the phylogenetic relationships of the subgroups studied.

For the phylogenetic analysis, we used the neighbor-joining (NJ; Saitou and Nei, 1987) and the maximum-parsimony (MP; Swofford and Olsen, 1990) methods. The numbers of nucleotide substitutions per site of COI and *Gpdh* were estimated by the Tamura and Nei (1993) method and the Kimura (1980) two-parameter model, respectively, by MEGA 1.0 (Kumar *et al.*, 1993). Those of the combined data set were estimated by the Kimura two-parameter model by MEGA 1.0. The statistical confidence of a particular cluster of sequences in the NJ trees was evaluated by the bootstrap test (1000 pseudoreplicates) by MEGA. The MP trees and their bootstrap tests (1000 pseudoreplicates) were obtained by the programs DNAPARS and SEQBOOT, respectively, implemented in the PHYLIP package 3.572 (Felsenstein, 1993). The partition homogeneity test was performed using PAUP 4.0 (Swofford, 1999).

Cold and Heat Tolerance

Cold tolerance of experimental flies was examined by two measures, half-lethal temperature when exposed to low temperature (LT_{50}) and duration of exposure to 1.5°C to kill half of the population (LD_{50}). LT_{50} was measured for experimental flies that were reared under a long daylength (15-h light:9-h dark) at 15°C to the 32-day adult stage and those that were reared under continuous light at 23°C to the 16-day adult stage. Flies were transferred to glass vials with food medium on the bottom and filter paper on the wall and directly exposed to low temperatures in constant darkness for 24 h. The food medium keeps moisture in the vials and the paper prevents flies from being caught by water drops on the wall. After the cold treatment, flies were placed at rearing temperature for 24 h and examined for survival. Usually two or three replicates were made for each experimental low temperature, and the survival rate was obtained from pooled data because usually no or little difference was observed in the survival rates among replicates. After survival rate was plotted against exposure temperatures, the temperatures that killed 25, 50, and 75% of population ($LT_{25,50,75}$) were obtained by directly reading intercepts of the curve. LD_{50} was measured for flies that were reared under a long daylength (15-h light:9-h dark) at 15°C to the 16-day adult stage: two replicates of 10–95 flies were placed in glass vials with food medium and exposed to 1.5°C in constant darkness, and survival was checked after 2, 4, 8, 12, 16, 24, 32, 48, and 64 days of exposure. Flies in chill-coma were allowed to recover at 15°C for 2 h before being checked. After being checked, they were returned to 1.5°C. After survival rate was plotted against duration of exposure, durations that killed 25, 50, and 75% of the population ($LD_{25,50,75}$) were obtained by directly reading intercepts of the curve.

Heat tolerance was examined by LT_{50}. The procedure was the same as that for the measurement of LT_{50} under cold treatment.

In this study, we examined LT_{50} under cold treatment in *D. constricta,* LT_{50} under heat treatment in *D. constricta, D. lutescens, D. takahashii, D. trilutea,* and *D. prostipennis,* and LD_{50} at 1.5°C in *D. rufa, D.*

TABLE 4

Percentage of A + T in All and/or Third Codon Position(s) in COI and *Gpdh*

Species	COI		*Gpdh*
	All	Third positions[a]	All
D. bifasciata	69.76	92.63 (93.55)	47.28
D. melanogaster	71.98	97.79 (96.77)	40.26
D. simulans	70.51	92.64 (91.94)	39.59
D. teissieri	70.02	91.90 (98.39)	39.58
D. lutescens	71.49	96.31 (100.00)	46.37
D. trilutea	70.26	92.64 (98.39)	45.24
D. prostipennis	69.77	91.90 (95.16)	46.37
D. takahashii	69.77	91.16 (98.39)	46.60
D. biauraria	71.25	97.05 (96.77)	39.81
D. triauraria	69.03	89.69 (90.32)	39.58
D. rufa	70.26	94.85 (95.16)	39.13
D. trapezifrons	69.77	91.17 (96.77)	39.13
D. watanabei	70.75	94.85 (98.39)	40.49
D. constricta	69.27	91.17 (91.94)	39.81

[a] A + T percentages of fourfold degenerate sites are shown in parentheses.

prostipennis, *D. constricta*, and *D. watanabei*. Data for the remaining species were reported by Kimura *et al.* (1994), Goto and Kimura (1998), Hori and Kimura (1998), and Ohtsu *et al.* (1998).

Ovarian Development under Long and Short Daylengths at 11°C

Animals were reared from eggs at 15°C before eclosion and 11°C after eclosion under long (15-h light:9-h dark) and short (10-h light:14-h dark) daylengths, and females were examined for ovarian development at the 32-day adult stage.

RESULTS

Phylogenetic Analyses of COI Sequences

We determined 407 bp of COI sequence and the first base corresponds to position 2205 in the *D. melanogaster* mtDNA sequence (the GenBank Accession No. is U37541). The COI fragments of 10 *Drosophila* species studied have a high proportion (69.03–71.49%) of A + T (Table 4), especially in third codon positions (89.69–97.79%) and at fourfold degenerate sites (90.32–100%), as has been reported for many other *Drosophila* species (DeSalle *et al.*, 1987; Nigro *et al.*, 1991; Tamura 1992; Beckenbach *et al.*, 1993; Gleason *et al.*, 1997; Goto *et al.*, 1999). Given the existence of substantive composition bias, we estimate nucleotide divergence according to the Tamura and Nei (1993) method.

Distances based on transversional substitutions range from 0.50 to 6.03% for comparisons within the subgroups and from 4.12 to 8.02% for comparisons between the subgroups (Table 5, above diagonal).

The ratio of transitions to transversions for all pairwise species comparisons ranges from 0.14 to 7.32 (Table 5, below diagonal). The highest ratios are between closely related species: for example, *D. biauraria–D. triauraria*. The lower ratios are for comparisons between the subgroups. A strong bias for transitional substitutions between closely related species, with a loss of this bias between more distantly related species, has been previously demonstrated for *Drosophila* mtDNA and has been explained by the fast saturation of transitional substitutions due to the strong biases in both base composition and substitution patterns (DeSalle *et al.*, 1987; Beckenbach *et al.*, 1993; Barrio *et al.*, 1994; Gleason *et al.*, 1997; Goto *et al.*, 1999). This is reflected in the following phenomenon: for transver-

TABLE 5

Percentage Transversional Substitution per Site (above Diagonal) and Transition/Transversion Ratio (below Diagonal) between COI Sequences Estimated According to the Tamura and Nei (1993) Method

Species	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.
1. *D. bifasciata*		7.44	6.87	7.16	5.20	5.20	5.48	5.20	5.75	5.75	7.44	6.87	7.44	6.59
2. *D. melanogaster*	0.43		**1.00**	3.32	5.20	5.20	4.93	5.20	6.87	6.31	6.87	4.12	5.20	7.16
3. *D. simulans*	0.51	**2.87**		3.32	4.66	5.20	4.93	5.20	6.87	6.31	6.87	4.66	5.20	6.59
4. *D. teissieri*	0.58	0.98	0.88		4.39	4.93	4.12	4.93	6.59	6.03	7.73	6.59	6.59	6.87
5. *D. lutescens*	0.56	0.36	0.33	0.42		**0.50**	**0.75**	**0.50**	6.87	6.87	8.02	6.31	6.31	6.59
6. *D. trilutea*	0.90	0.57	0.62	0.59	**3.06**		**0.75**	**0.50**	6.03	6.03	7.73	6.03	6.31	6.31
7. *D. prostipennis*	0.82	0.73	0.71	0.78	**2.77**	**2.05**		**0.75**	6.03	6.03	7.73	6.03	6.03	6.31
8. *D. takahashii*	0.90	0.63	0.67	0.66	**3.62**	**3.58**	**3.10**		6.87	6.87	8.02	6.31	6.31	7.16
9. *D. biauraria*	0.62	0.14	0.43	0.59	0.40	0.56	0.71	0.62		**0.50**	**2.53**	**2.53**	3.58	4.93
10. *D. triauraria*	1.03	0.60	0.93	1.04	0.78	0.78	1.15	0.91	**7.32**		**2.02**	**2.02**	3.05	4.39
11. *D. rufa*	0.51	0.43	0.55	0.46	0.40	0.63	0.62	0.67	**1.26**	**2.40**		**2.53**	3.58	6.03
12. *D. trapezifrons*	0.82	0.99	1.06	0.77	0.81	0.89	1.05	0.85	**1.72**	**2.67**	**1.69**		**2.02**	4.39
13. *D. constricta*	0.52	0.76	1.04	0.90	0.68	0.92	1.02	0.67	1.26	2.00	1.40	**2.75**		3.32
14. *D. watanabei*	0.57	0.41	0.40	0.55	0.27	0.45	0.65	0.53	0.71	1.32	0.44	1.19	1.31	

Note. Comparisons with transversional divergences less than 3% are shown in boldface.

sional divergences less than 3%, the mean Ti/Tv ratio is 2.97, whereas for divergences greater than 4%, the ratio is 0.73. This suggests that transitional substitutions have reached saturation for these species comparisons and, therefore, for the analyses of COI, we used only transversions.

Figure 1 shows the phylogenetic tree based on COI by NJ using only transversions. Each subgroup formed a cluster. In the *takahashii* subgroup, *D. prostipennis* (subtropical highland species) branched off first, and *D. lutescens* (warm-temperate species) did next. In the *montium* subgroup, *D. triauraria* (cool-temperate species) formed a cluster with *D. biauraria* (cool-temperate species) and this cluster was combined with *D. rufa* (warm-temperate species). In this subgroup, *D. watanabei* (subtropical lowland species) branched off first.

Phylogenetic Analyses of Gpdh Sequences

The sequence of the *Gpdh* gene analyzed in this study was 442 bp in length (69 bp from exon 3 and 373 bp from exon 4). There is little bias in A + T content in the *Gpdh* sequences (Table 4): the average of overall nucleotide frequencies are 0.219 T, 0.270 C, 0.203 A, and 0.307 G. Given the absence of substantive composition bias, we estimate nucleotide divergence according to the Kimura (1980) two-parameter model.

Figure 2 shows the phylogenetic tree based on *Gpdh* by NJ. Each subgroup formed a cluster. In the *takahashii* subgroup, *D. takahashii* branched off next to *D. prostipennis*. In the *montium* subgroup, the topology was similar to the tree derived from COI.

Phylogenetic Analyses with the Combined Data Set

To compare the trees derived from COI and *Gpdh*, the partition homogeneity test was performed. Since the *P* value was 0.298 between COI and *Gpdh*, the combined data set was used to construct the NJ and MP trees. Since the base frequencies were 0.250 A, 0.307 T, 0.203 C, and 0.240 G, the Kimura two-parameter model (Kimura, 1980) was used. Both NJ and MP trees were identical in topology. Therefore, we present only the NJ tree in Fig. 3. In the *takahashii* subgroup, *D. lutescens* (warm-temperate species) branched off first, and *D. prostipennis* (subtropical highland species) did next. In the *montium* subgroup, the topology was identical to the trees derived from *Gpdh*.

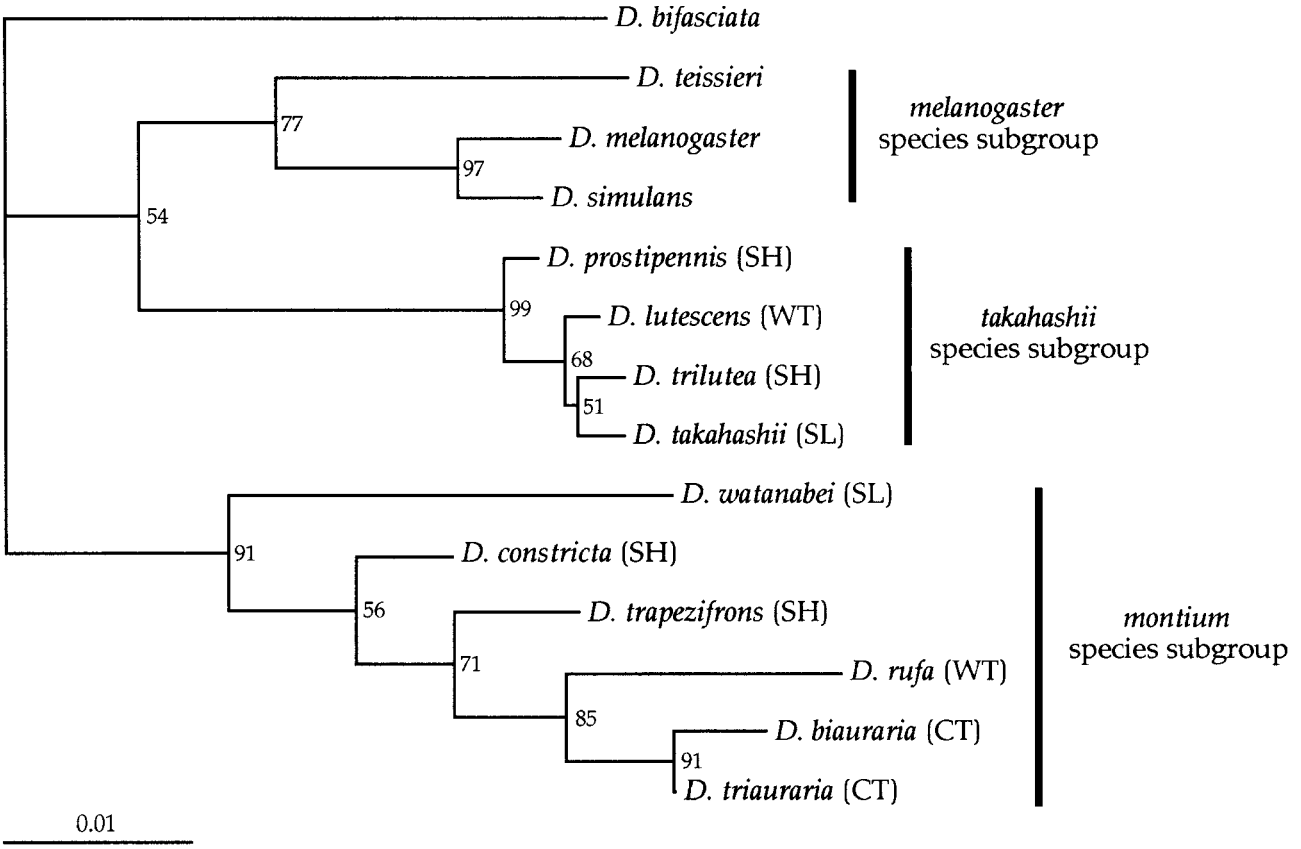

FIG. 1. Neighbor-joining tree based on COI sequences using only transversions. Branch lengths are proportional to the scale given in substitutions per nucleotide. Bootstrap values (percentage of 1000 pseudoreplicates) are shown at each node. *D. bifasciata* was used as an outgroup. Ecotypes of the experimental species are indicated in parentheses; SL, subtropical lowlands; SH, subtropical highlands; WT, warm-temperates; and CT, cool-temperates.

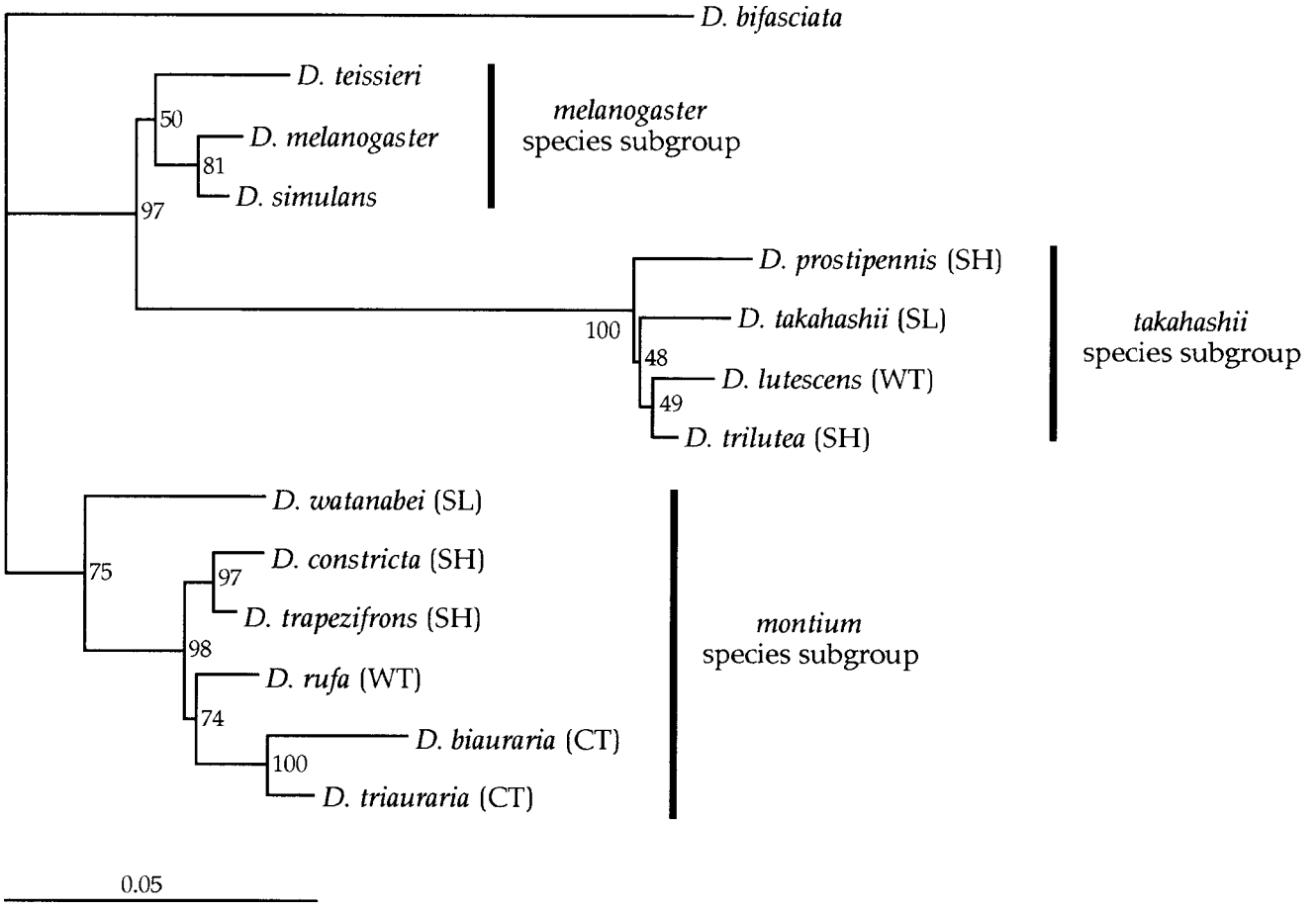

FIG. 2. Neighbor-joining tree based on *Gpdh* sequences using all substitutions. For further explanations, see the legend to Fig. 1.

Temperature Tolerance

The cool-temperate species *D. biauraria* and *D. triauraria* had very low LT_{50} compared to that of the others (Fig. 4). For these species, LD_{50} was not obtained because they survived very long at 1.5°C. The warm-temperate species *D. lutescens* and *D. rufa* were less cold tolerant than the cool-temperate species but much more cold tolerant than the subtropical highland and lowland species (Figs. 4 and 5). Among the subtropical species, *D. trapezifrons*, *D. trilutea*, and *D. prostipennis* (the highland species) survived somewhat longer than *D. watanabei* and *D. takahashii* (the lowland species) at 1.5°C, but LT_{50} differed little between them (Figs. 4 and 5). *D. constricta* (a highland species) was as cold susceptible as were the subtropical lowland species (Figs. 4 and 5). The subtropical highland species were generally less heat tolerant than the subtropical lowland and temperate species (Fig. 4). The decrease in rearing temperature lowered LT_{50} under cold treatment in all species but had no or little effects on LT_{50} under heat treatment (Fig. 4).

Ovarian Development under Long and Short Daylengths at 11°C

In the warm-temperate species, ovarian development was retarded under a short daylength at 11°C but not under a long daylength (Table 6). Such a response to photoperiod was not observed in the subtropical highland and lowland species. In the subtropical lowland species (*D. watanabei* and *D. takahashii*), ovarian development was retarded at 11°C, irrespective of photoperiod.

DISCUSSION

East Asian species of the *D. montium* and *takahashii* subgroups are subdivided into four groups, cool-temperate, warm-temperate, subtropical highland, and subtropical lowland species, in the present and previous studies (Kimura, 1984, 1988; Kimura *et al.*, 1994; Ohtsu *et al.*, 1993, 1998; Goto and Kimura, 1998; Hori and Kimura, 1998). The cool-temperate species (*D. biauraria*, *D. triauraria*, and their siblings) are charac-

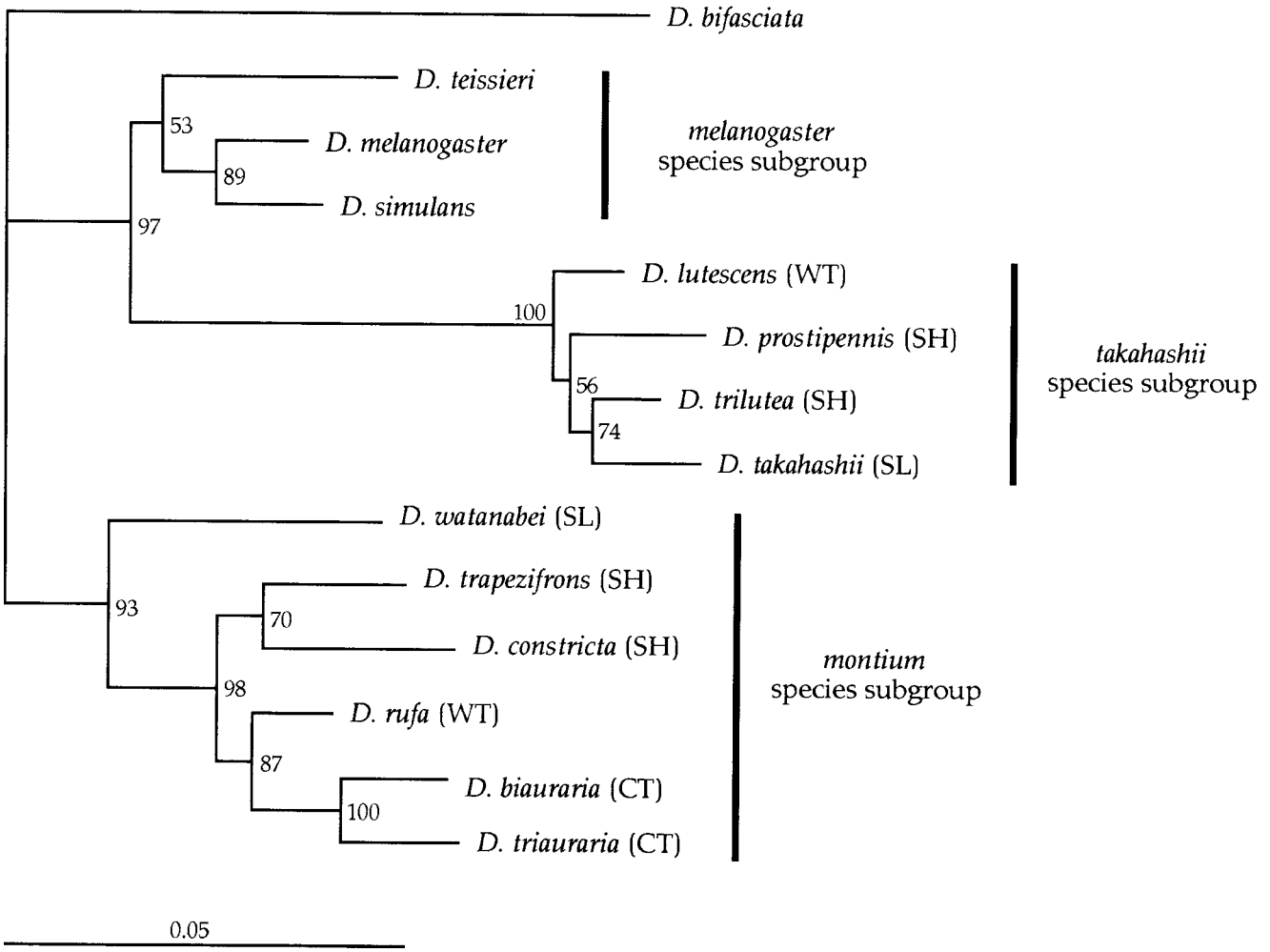

FIG. 3. Neighbor-joining tree based on the combined data set of COI and *Gpdh*. For further explanations, see the legend to Fig. 1.

terized by firm reproductive diapause and very high cold tolerance, the warm-temperate species (*D. rufa* and *D. lutescens*) by shallow reproductive diapause and moderately high cold tolerance, the subtropical highland species (*D. trapezifrons, D. constricta, D. prostipennis,* and *D. trilutea*) by low tolerance to both cold and heat (but most of them are slightly more cold tolerant than the subtropical lowland species), and the subtropical lowland species (*D. watanabei* and D. *takahashii*) by high heat tolerance, low cold tolerance, and low ability to reproduce at moderately low temperature (e.g., 11°C).

The present analysis based on the combined data of COI and *Gpdh* suggests that the ancestor of the *takahashii* subgroup split into warm-temperate and subtropical species first and subtropical species subsequently split into subtropical highland and subtropical lowland species. However, the nucleotide divergence among them was small and the phylogenetic trees based on single genes differ in topology from the tree based on the combined data. In addition, Inaba *et al.* (1993) and Parkash *et al.* (1994) reported that the phylogenetic trees in this subgroup based on restriction analyses and protein differences do not always coincide. Moreover, the members of this subgroup are morphologically very similar (Lemeunier *et al.*, 1986) and produce interspecific hybrids (Kimura, 1982; Inaba *et al.*, 1993). Thus, phylogenetic relationships in this subgroup are still ambiguous. Extensive studies would be required to resolve the phylogenetic relationships of these species.

The *auraria* species complex of the *montium* subgroup first consisted of five species, *D. auraria* Peng, *D. biauraria, D. triauraria, D. quadraria* Bock and Wheeler, and *D. subauraria* Kimura (Lemeunier *et al.*, 1986), and later Kim *et al.* (1989, 1993) included *D. rufa* and its siblings in this complex. The first five species (henceforth called the *auraria* lineage) were subdivided into two clades, *auraria–triauraria–quadraria* and *biauraria–subauraria,* by genetical, biochemical, and molecular studies (Ohnishi and Watanabe, 1984; Kimura,

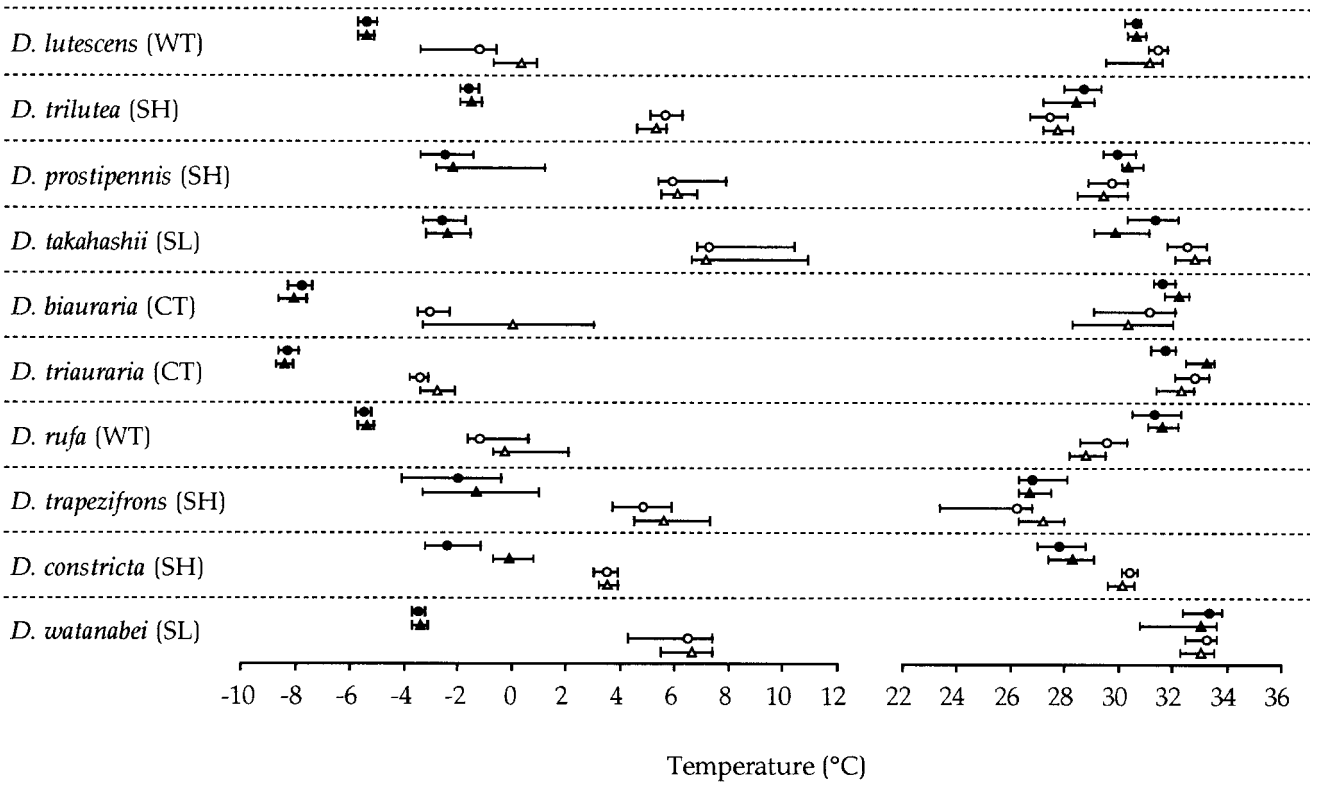

FIG. 4. Half-lethal temperature of experimental species reared under a long daylength (15-h light:9-h dark) at 15°C (●, ▲) and under continuous light at 23°C (○, △), when they were exposed to high and low temperatures for 24 h. ●, ○: female. ▲, △: male. The ends of bars show LT_{25} and LT_{75}. For abbreviations, see the legend to Fig. 1.

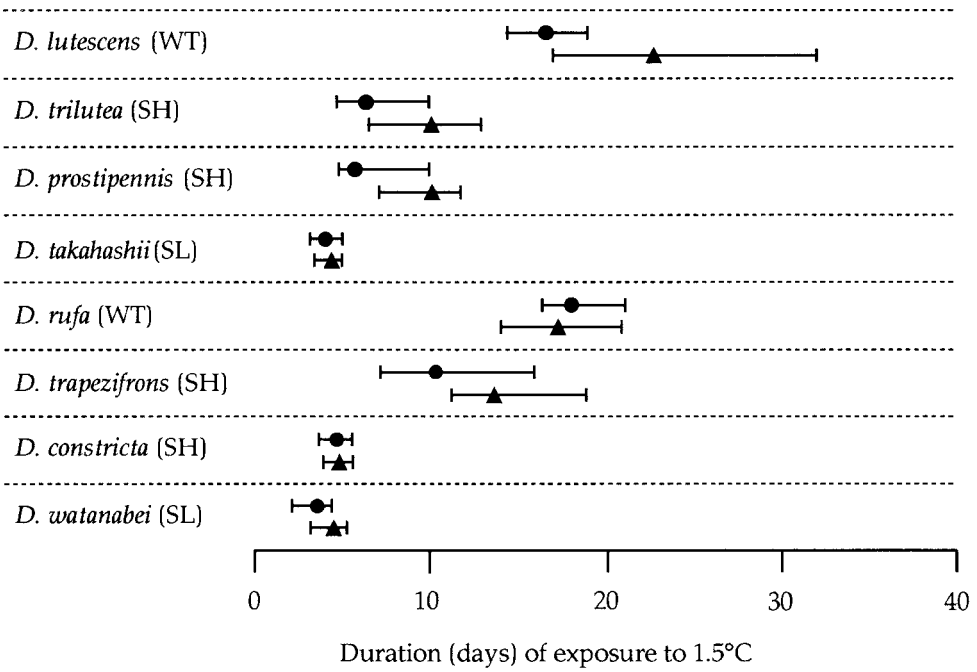

FIG. 5. Duration of exposure to 1.5°C to kill half of the population (LD_{50}) in experimental species reared under a long daylength (15-h light:9-h dark) at 15°C. ●: female, ▲: male. The ends of bars show LD_{25} and LD_{75}. For abbreviations, see the legend to Fig. 1.

TABLE 6

Percentage of Females with Mature Ovaries under Long (15-h Light: 9-h Dark) and Short (10-h Light: 14-h Dark) Daylengths

Species	Daylength	
	Long	Short
D. lutescens (WT)	97.4 (35)	2.0 (50)
D. trilutea (SH)	98.2 (54)	100.0 (27)
D. prostipennis (SH)	98.7 (70)	100.0 (37)
D. takahashii (SL)	38.0 (50)	40.0 (45)
D. rufa (WT)	85.7 (27)	0.0 (35)
D. trapezifrons (SH)	100.0 (17)	100.0 (23)
D. constricta (SH)	100.0 (25)	88.2 (17)
D. watanabei (SL)	0.0 (32)	0.0 (35)

Note. Individuals were reared at 15°C before eclosion and 11°C after eclosion, and ovarian development was examined 32 days after eclosion. Numbers in parentheses refer to number of females examined. Ecotypes of the experimental species are indicated on the right of species name in parentheses; WT, warm-temperates; SH, subtropical highlands; and SL, subtropical lowlands.

1987; Kim *et al.*, 1993). In both clades, firm reproductive diapause and very high cold tolerance are common, suggesting that the ancestor of the *auraria* lineage had adapted to cool-temperate climates (Kimura, 1988; Ohtsu *et al.*, 1998).

On the other hand, Ohnishi *et al.* (1983), Ohnishi and Watanabe (1984), and Kim *et al.* (1993) suggested that *D. rufa* (a warm-temperate species) forms a cluster with four species, *D. tani* Cheng and Okada, *D. lacteicornis* Okada, *D. asahinai* Okada, and *D. yuwaensis* Kim and Okada (but the last species would be conspecific to *D. asahinai;* M. T. Kimura, unpublished). *D. tani* occurs from northern subtropics to southern temperates (Cheng and Okada, 1985), and *D. lacteicornis* and *D. asahinai* occur in islands located in northern subtropics. It is therefore considered that the *auraria–rufa* lineage (i.e., the *auraria* complex) evolved in warm-temperates or northern subtropics and thereafter the *auraria* lineage evolved along with adaptations to cool-temperate climates.

The *auraria–rufa* lineage forms a cluster with *D. trapezifrons* and *D. constricta,* subtropical highland species. In this cluster, one more species, *D. khaoyana* Bock and Wheeler, which is close to *D. constricta* (Chen *et al.*, 1988), is known. According to our preliminary study, this species is assumed to inhabit subtropical highlands with *D. constricta.* On the other hand, Ohnishi *et al.* (1983) and Kim *et al.* (1993) suggested that *D. watanabei* (cited as *punjabiensis*-like) is a member of the *jambulina* species complex, which forms a cluster with the *kikkawai* complex. It has been reported that these two complexes have diversified in Asian tropics (Lemeunier *et al.*, 1986). This information seems to support a notion that the warm-temperate species derived from subtropical highland species, at least in the *montium* subgroup.

In comparison with the subtropical lowland species, the subtropical highland species may be better able to colonize temperate areas since, as in the temperate species, they have an ability to develop their ovaries at moderately low temperature (11°C). However, the subtropical highland species, as well as the subtropical lowland species, were much less cold tolerant than the temperate species. Therefore, considerable genetic reformation would be required for both the subtropical highland and the subtropical lowland species to adapt to temperate climates.

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