Evolution of overwintering strategies in Eurasian species of the \textit{Drosophila obscura} species group

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The phylogenetic relationship of Eurasian species of the \textit{Drosophila obscura} species group remains ambiguous in spite of intensive analyses based on morphology, allozymes and DNA sequences. The present analysis based on sequence data for cytochrome oxidase subunit I (COI) and \(\alpha\)-glycerophosphate dehydrogenase (Gpdh) suggests that the phylogenetic position of \textit{D. alpina} is also ambiguous. These ambiguities have been considered to be attributable to rapid phyletic radiation in this group at an early stage of its evolution. Overwintering strategies are diversified among these species: \textit{D. alpina} and \textit{D. subsilvestris} pass the winter in pupal diapause, \textit{D. bifasciata} and \textit{D. obscura} in reproductive diapause, and \textit{D. subobscura} and \textit{D. guanche} without entering diapause. This diversity may also suggest rapid radiation at an early phase of adaptations to temperate climates. On the other hand, adult tolerance of cold was closely related to overwintering strategy and distribution: \textit{D. obscura} and \textit{D. bifasciata} with reproductive diapause were very tolerant; \textit{D. alpina} and \textit{D. subsilvestris} which pass the winter in pupal diapause were less tolerant; \textit{D. subobscura} having no diapause was moderately tolerant and \textit{D. guanche} occurring in the Canary Islands was rather susceptible. Tolerance of high temperature at the preimaginal stages seemed to be also associated with overwintering strategy; i.e. lower in the species with pupal diapause than in those with reproductive diapause or without diapause mechanism.

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ADDITIONAL KEY WORDS:—diapause — molecular phylogeny — temperature tolerance.
INTRODUCTION

The *Drosophila obscura* species group has been the subject of many studies on evolutionary phenomena, for example, reproductive isolation, mating preference, and inversion polymorphism and genetic variation in natural populations (reviewed in Dobzhansky & Powell, 1975; Lakovaara & Saura, 1982; Powell, 1997). The phylogeny of this species group has also been extensively studied on the basis of morphology, chromosomes, allozymes and DNA sequences of several genes. These studies identified two subgroups, *affinis* and *pseudoobscura*, in the North American species, and two clades, *obscura* and *subobscura*, plus two other species (*D. bifasciata* Pomini and *D. subsitwesr* Hardy & Kaneshiro) with unresolved phylogenetic positions in the Eurasian species (Lakovaara & Saura, 1982; Beckenbach, Wei & Liu, 1993; Pelandakis & Solignac, 1993; Russo, Takezaki & Nei, 1995; Gleason et al., 1997; Barrio & Ayala, 1997). It has also been suggested with allozyme data that *D. alpina* Burla is not appreciably related to any subgroups (Lakovaara et al., 1976), but its phylogenetic position has not been analysed by molecular data. Thus, ambiguities still remain on the phylogenetic relationship among the Eurasian species. Barrio & Ayala (1997) and Gleason et al. (1997) considered that these ambiguities are attributable to rapid phyletic radiation at an early phase of the evolution of this group. Here we report the phylogenetic position of *D. alpina* based on sequence data of COI (mitochondrial) and *Gpdh* (nuclear) genes and also add data on overwintering strategies (diapause and temperature tolerance) for the Eurasian species of this group.

Seasonality is an important factor shaping the evolution of temperate and arctic insects (Lumme & Lakovaara, 1983; Tauber, Tauber & Masaki, 1986; Danks, 1987). In fact, the acquisition of overwintering abilities has been suggested to play a key role in the evolution of temperate species in the *Drosophila melanogaster* species group (Kimura, 1988; Kimura et al., 1994; Ohtsu et al., 1993; Ohtsu, Kimura & Katagiri, 1998). In the *obscura* group, information on overwintering strategies would be also useful to understand its adaptations in temperate regions and resolve its speciation history. It has been reported that the members of the *obscura* group vary in their
seasonal life histories; *D. obscura* Fallén and *D. bifasciata* have reproductive diapause and pass the winter as adults, *D. subobscura* Collin has no diapause, while *D. alpina* pass the winter in pupal diapause (Begon, 1976; Lumme & Lakovaara, 1983; Toda et al., 1986; Beppu, Yoshida & Kimura, 1996).

**MATERIAL AND METHODS**

**Flies**

The experimental strains used originated as follows: *D. obscura* from 10–20 females collected in Tübingen (Germany) in 1993; *D. subobscura* from several females collected at Heuberger Tor (near Tübingen) in 1993; *D. guanche* Monclus from an old strain collected in the 1970s from the Canary Islands; *D. subsilvestris* from a single female collected at Heuberger Tor in 1992; *D. alpina* from several females collected in Shiga (central Japan) in 1993; *D. bifasciata* from females collected in Sapporo (SP: Japan) and Yakutsk (YK: Siberia, Russia) in 1993. Except for *D. guanche*, diapause and temperature tolerance were examined within one or two year(s) following the collection of the strains. Cornmeal-malt medium was used for rearing.

**Sequencing of COI and Gpdh genes from *D. alpina***

To perform PCR, DNA was extracted from *D. alpina* according to the method of Goto, Yoshida & Kimura (1998). RNA in the samples was digested with RNase A.

The primers used to amplify COI were designed according to Gleason et al. (1997); F-COI, 5’> CCAGC TGGAG GAGGA GATCC >3’; R-COI, 5’> CCAGT AAATA ATGGG TATCA GTG>3’. In addition, we designed the primer on the basis of the sequence derived from *D. takahashii* (Nigro, Solignac & Sharp, 1991) for nested PCR; takl, 5’> GCITG AGCCG GAATA GTAGG >3’. The first base of takl corresponds to position 1537 in the *D. melanogaster* Meigen mtDNA sequence (the Genbank accession number is U37541). Primary reaction of nested PCR used 100 ng of DNA, 1 U of AmpliTaq DNA polymerase (Perkin-Elmer), and final concentration of 1.5 mM of MgCl2, 1 × PCR buffer II as formulated by Perkin-Elmer, 0.3 µM of takl 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 sec denaturing at 94°C, 30 sec annealing at 50°C, and 45 sec extension at 72°C. Secondary reaction used the same components of the primary reaction, except that the primers were F-COI and R-COI, and the target was 1 µl of the primary amplified product. PCR conditions were the same as above. The length of amplified products agreed with the one of COI reported by Gleason et al. (1997).

**Gpdh** fragment was amplified with the primer set of GNL and GNR (Barrio & Ayala, 1997). PCR components were the same as those used for amplification of the COI region, except the primers. Amplification was performed with 35 cycles of 30 sec denaturing at 94°C, 30 sec annealing at 56°C, and 45 sec extension at 72°C. Secondary reaction used the same components of the primary reaction, except that the primers were F-COI and R-COI, and the target was 1 µl of the primary amplified product. PCR conditions were the same as above. The length of amplified products agreed with the one of COI reported by Gleason et al. (1997).

The amplified fragments were purified from the gel using Prep-A-Gene DNA purification kit (BIO-RAD). The sequences were obtained from an ABI 373A
automated sequencer (PE Applied Biosystems) with DNA sequencing kit (Dye Terminator Cycle Sequencing Ready Reaction; PE Applied Biosystems) according to supplier’s instructions. Cycle sequencing was performed using two oligonucleotides (F- and R-COI) and six (GNL, GNR, L4BN, L4E, R5B and R4M; Barrio & Ayala, 1997) for COI and Gpdh fragments, respectively. The obtained nucleotide sequence data of D. alpina were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB016601 and AB016727 for COI and Gpdh, respectively.

The species name and the Genbank accession numbers for the COI and Gpdh sequences used for the phylogenetic analysis are as follows (two numbers are for the COI and Gpdh sequences, respectively): Drosophila melanogaster (U37541, X14179); D. affinis Sturtevant (U51604, U47874); D. madeirensis Monclus (U51613, U47890); D. guanche (U51612, U47878); D. ambigua Pomini (U51610, U47880); D. obscura (U51614, U47881); D. subsilvestris (U51616, U47884); D. azteca Sturtevant and Dobzhansky (U51605, U47875); D. miranda Dobzhansky (U51608, U47882); D. pseudoobscura bogotana Ayala & Dobzhansky (U51607, U47891); D. subsobscura (U51615, U47877); D. pseudoobscura pseudoobscura Frolova (U51606, U47883); D. persimilis Dobzhansky & Epling (U51609, U47886); D. bifasciata (U51611, U47883).

**Phylogenetic inference**

The sequences were aligned using the CLUSTAL V program (Higgins & Sharp, 1988). The length of the COI sequence was 462 bp and the first base corresponds to position 2167 in the D. melanogaster mtDNA sequence (the Genbank accession number is U37541). On the other hand, the first and last bases of the Gpdh sequences correspond to position 3036 and 4230 in the D. melanogaster gene (the Genbank accession number is X14179), respectively.

For phylogenetic analysis, we used the neighbour-joining (NJ; Saitou & Nei, 1987) and the maximum-parsimony (MP; Swofford & Olsen, 1990) methods. The numbers of nucleotide substitutions per site of COI and Gpdh were estimated by Tamura & Nei’s (1993) method and Kimura’s (1980) two-parameter model, respectively, by MEGA 1.0 (Kumar, Tamura & Nei, 1993). 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 (500 pseudoreplicates) were obtained by the programs DNAPARS and SEQBOOT, respectively, implemented in PHYLIP ver. 3.572 (Felsenstein, 1993). Since the introns were so diversified among the species studied, the gaps and missing information in the sequences were aligned with the pairwise-deletion option in MEGA.

**Diapause and temperature tolerance**

Experimental individuals were reared from eggs and exposed to short (10 h-light/14 h-dark) and long (15 h-light/9 h-dark) daylengths at 15°C and examined for eclosion. For species which eclosed at these photoperiods, ovarian development was examined 16 days after eclosion; females with undeveloped ovaries were classified as being in reproductive diapause. In addition, flies were examined to ascertain whether they were able to complete preimaginal development at 15, 21, 25.5 and
29°C (exposed to long daylength or continuous illumination): about 100 eggs were placed in vials containing 10 ml food medium (about 50 eggs per vial) and examined for the rate of pupation (D. alpina and D. subsilvestris) or eclosion (the other species). To examine tolerance of cold, the experimental species except D. alpina were reared from the egg stage, exposed to short and long daylengths at 15°C, continuous illumination at 21°C and examined for survival at low temperatures 16 days after eclosion. In D. alpina, which entered pupal diapause irrespective of photoperiod, the experimental protocol was as follows: individuals were reared from the egg stage, exposed to a short daylength at 15°C; diapausing pupae thus obtained and aged 2 months after being oviposited were reared exposed to 4°C (constant darkness) for 2.5 months; these cold-treated pupae were exposed to a long daylength at 15°C and adults eclosed from these pupae were examined for cold tolerance 16 days after eclosion. Experimental flies were introduced into vials containing food medium and exposed to low temperature (−1 to −15°C) for 24 h (about 30 individuals at each temperature). Survival was examined after flies were maintained at 15°C for 24 h after cold treatment. Temperatures that killed 25, 50 and 75% of populations (LT$_{25,50,75}$) were obtained by reading the intercepts after survival was plotted against exposure temperature.

RESULTS

Phylogenetic analyses of COI sequences

The fragment of the COI gene of D. alpina has a high proportion (71.4%) of A+T, especially in third codon positions (95.4%) and at fourfold degenerate sites (100%), as has been reported for the other members of the obscura species group and also for many other Drosophila species (DeSalle et al., 1987; Nigro et al., 1991; Tamura, 1992; Beckenbach et al., 1993; Barrio, Latorre & Moya, 1994; Gleason et al., 1997). Given the existence of substantive composition bias, we estimate nucleotide divergence according to Tamura & Nei’s (1993) method.

The ratio of transitions to transversions (Ti/Tv) between D. alpina and the other species ranges from 0.471 (D. affinis) to 1.301 (D. guanche) (Table 1). In the obscura group, Gleason et al. (1997) reported that the Ti/Tv ratio ranges from 0.29 to 3.68 and is generally high between closely related species and low between distantly related species. This trend has been explained by the fast saturation of transitional substitutions (DeSalle et al., 1987; Gleason et al., 1997). We used only transversions for the phylogenetic analyses, following Gleason et al. (1997).

The number of transversional substitutions per site between D. alpina and the other species ranges from 0.0411 (D. guanche) to 0.0655 (D. pseudoobscura and some other North American species) (Table 1). Figure 1 shows the results of phylogenetic analysis based on COI by NJ. The analysis suggests that D. alpina splits first in the obscura species and is not clustered in any subgroups. The same result was obtained in the analysis by MP.

Phylogenetic analyses of Gpdh sequences

The sequence of the Gpdh gene of D. alpina analysed in this study was 965 bp in length and distributed as follows: 152 bp from exon 3, 77 bp from intron III, 373 bp
Table 1. Pairwise nucleotide divergences for only transversions (Tv) and transition/transversion ratio (Ti/Tv), and for Ti/Tv and all substitutions [Al] between COI and Gpdh exon sequences of D. alpina and ones of the other species, respectively

| Species             | COI   | Gpdh  |
|---------------------|-------|-------|
|                     | Ti/Tv | Tv    | Ti/Tv | Al   |
| D. melanogaster     | 0.5147| 0.0459| 2.9719| 0.1522|
| D. subobscura       | 1.1846| 0.0581| 1.6330| 0.0365|
| D. madeirensis      | 0.7643| 0.0581| 1.4839| 0.0379|
| D. guanche          | 1.3014| 0.0411| 1.7819| 0.0307|
| D. ambiguus         | 0.9335| 0.0556| 2.6459| 0.0251|
| D. obscura          | 1.2220| 0.0532| 2.4388| 0.0237|
| D. subsilvestris    | 0.9838| 0.0381| 1.5933| 0.0250|
| D. bifasciata       | 0.7289| 0.0459| 2.0317| 0.0251|
| D. affinis          | 0.4709| 0.0655| 1.7939| 0.0358|
| D. azteca           | 0.4715| 0.0655| 1.9063| 0.0348|
| D. miranda          | 0.6101| 0.0630| 2.9675| 0.0438|
| D. p. pseudobscura  | 0.7230| 0.0655| 2.6372| 0.0433|
| D. p. bogotana      | 0.6012| 0.0655| 2.6372| 0.0433|
| D. persimilis       | 0.7607| 0.0655| 2.8342| 0.0424|

The divergence of COI and Gpdh sequences were estimated according to Tamura and Nei’s (1993) method and Kimura’s (1980) two-parameter model, respectively.

![Neighbour-joining tree based on COI sequences using only transversion](image)

Figure 1. Neighbour-joining tree based on COI sequences using only transversion. Branch lengths are proportional to the scale given in substitutions per nucleotide. Bootstrap values (percentage of 1000 pseudoreplicates) are shown at each node.

from exon 4, 67 bp from intron IV, 154 bp from exon 5, 89 bp from intron V and 53 bp from exon 6. Due to the difficulty of unambiguously aligning the Gpdh intron sequences, we examined separately the exon and the intron sequences.
There is little bias in G+C content in the \textit{Gpdh} exon sequences: the averages of the nucleotide frequencies are 0.242 A, 0.232 C, 0.272 G and 0.254 T. Given the absence of substantive composition bias, we estimate nucleotide divergence according to Kimura’s (1980) two-parameter model (Table 1). Divergence between \textit{D. alpina} and the other species in all substitutions ranges from 0.0237 (\textit{D. obscura}) to 0.1522 (\textit{D. melanogaster}). The topologies of the NJ and MP trees are identical except the position of \textit{D. alpina}. In the NJ tree, \textit{D. alpina} is placed at the root of the cluster of the Eurasian species (Fig. 2A), but at the root of the \textit{subobscura} subgroup with lower bootstrap value in the MP tree (not shown).

In the NJ tree based only on the intron sequences (which does not include the outgroup \textit{D. melanogaster}), \textit{D. alpina} is placed at the root of the \textit{D. subsilvestris} and \textit{D. bifasciata} clusters with a lower bootstrap value (Fig. 2B). In the MP tree, \textit{D. alpina} is diverged from the root of the cluster of the Eurasian species with a bootstrap value of 96 (not shown).

\textit{Photoperiodic control of diapause}

In \textit{D. alpina}, almost all individuals which developed to pupae did not eclose in 2 months after being oviposited irrespective of photoperiod (Table 2 and Fig. 3). In \textit{D. subsilvestris}, most of the pupae did not eclose in the two months after being oviposited and exposed to a short daylength, but about 74% of pupae eclosed with a long daylength (Table 2). When these pupae were maintained at 4°C for 2–4 months and then brought into an environment at 15°C, a number of them eclosed (data not shown), indicating that these pupae were in diapause. When \textit{D. alpina}
Table 2. Percentage of individuals in pupal and reproductive diapause under short and long daylengths at 15°C. The individual of pupal diapause was examined 2 months after being oviposited and that of reproductive diapause was examined 16 days after eclosion.

|                                      | Short daylength | Long daylength |
|--------------------------------------|-----------------|----------------|
| Pupal diapause                       |                 |                |
| *D. subsilvestris*                   | 98.2 (57)       | 26.2 (42)      |
| *D. altina*                          | 100 (5)         | 100 (50)       |
| Reproductive diapause                |                 |                |
| *D. bifasciata SP*                   | 100 (32)        | 50.0 (18)      |
| *D. bifasciata YK*                   | 64.2 (67)       | 30.0 (10)      |
| *D. obscura*                         | 69.9 (73)       | 0 (52)         |
| *D. subobscura*                      | 0 (14)          | 0 (14)         |
| *D. guanche*                         | 0 (29)          | 0 (16)         |

Figures in parentheses refer to number of individuals examined.

Pupae were maintained at 15°C further, some of them eclosed 3–6 months after being oviposited with a long daylength, although almost none of them eclosed with a short daylength (Fig. 3).

In *D. bifasciata* and *D. obscura*, the percentage of females in reproductive diapause was significantly ($\chi^2$-test, $P<0.05$) higher at a short daylength than at a long daylength (Table 2). On the other hand, *D. subobscura* and *D. guanche* showed no sign of photoperiodic diapause.

Temperature tolerance

Preimaginal development was retarded at 29°C in *D. bifasciata*, *D. obscura*, *D. subobscura* and *D. guanche*, but at a lower temperature, 25.5°C, in *D. altina* and *D. subsilvestris* (Fig. 4).

Half lethal temperature ($\text{LT}_{50}$) for adult flies of the experimental species is shown in Figure 5. In all species, $\text{LT}_{50}$ was lower in flies reared at 15°C than in those reared at 21°C. In *D. bifasciata* and *D. obscura*, flies reared at a short daylength...
Figure 4. Viability from egg to pupariation in *D. alpina* (●) and *D. subsilvestris* (●) and from egg to eclosion in *D. bifasciata* (SP: ○, YK: ◇). *D. obscura* (△), *D. subobscura* (▽) and *D. guanche* (□).

Figure 5. Cold tolerance of females of experimental species reared under short (○) and long daylength (●) at 15°C and under continuous illumination at 21°C (○). Symbols indicate half lethal temperature (*LT*₅₀) and horizontal lines indicate *LT*₂₅ and *LT*₇₅. Males differed little from females in lethal temperatures.

usually had lower *LT*₅₀ than those reared at a long daylength: the difference in survival rate between individuals reared at short and long daylengths was significant (*χ²*-test, *P*<0.01), at least at one temperature regime.
In *D. bifasciata*, the Yakutsk strain exhibited more tolerance of cold than the Sapporo strain, while *D. obscura* was as tolerant as the Yakutsk strain. On the other hand, *D. subobscura* exhibited less tolerance than the above two species, and *D. guanche* was rather susceptible to cold. The other two species, *D. alpina* and *D. subsilvestris*, displayed less tolerance, but more than *D. guanche*.

**DISCUSSION**

*The phylogenetic position of D. alpina*

Previous molecular studies (Beckenbach *et al.*, 1993; Pelandakis & Solignac, 1993; Russo *et al.*, 1995; Gleason *et al.*, 1997; Barrio & Ayala, 1997) reported that the phylogenetic relationship among the Eurasian members of the *obscura* species group is ambiguous. The present study also suggested that the phylogenetic position of *D. alpina* is ambiguous: its position differed by genes or methods used for the analysis. Gleason *et al.* (1997) and Barrio & Ayala (1997) considered that these ambiguities are attributable to rapid phyletic radiation at an early phase of the evolution of this group. They also identified the *obscura–tristis–ambigua* and *subobscura–guanche–madeirensis* subgroups plus two other species with unresolved phylogenetic positions, *D. bifasciata* and *D. subsilvestris*, in the Eurasian members of the *obscura* species group.

The present analysis based on the sequence data of COI and *Gpdh* revealed that *D. alpina* does not cluster with any other members of this species group. Lakovaara *et al.* (1976) also suggested with allozyme data that *D. alpina* is not appreciably related to any cluster. *D. alpina* is also distinct in this species group in morphology and chromosomal composition (Lakovaara & Saura, 1982). It is therefore assumed that *D. alpina* is derived from an early radiation of this species group.

*Evolution of overwintering strategies*

Throckmorton (1975) suggested, based on comparison of morphological characters, that the *obscura* species group is most closely related to the *melanogaster* species group. This notion is supported by the cladistic analysis of morphological characters (Grimaldi, 1990) and also by molecular data (Pelandakis & Solignac, 1993; Russo *et al.*, 1995). At present, the *melanogaster* species group shows major radiation in tropical regions (Lemeunier *et al.*, 1983), with the *obscura* species group predominant in temperate forests (Lakovaara & Saura, 1982). Based on the view that drosophilids are of tropical origin (Throckmorton, 1975), it is assumed that the *melanogaster* group retains ancestral characteristics and that the ancestor of the *obscura* group split from the *melanogaster* group following its colonization of temperate regions.

It is considered that the *subobscura* subgroup displays an ancestral characteristic in climatic adaptations (lack of diapause), since tropical species of the *melanogaster* group do not have a diapause mechanism (Kimura, 1988; Kimura *et al.*, 1994). However, there is also the possibility that the *subobscura* subgroup has lost the diapause mechanism which had once evolved in conjunction with its colonization of temperate regions. Kimura (1988) reported such a case in *D. triauraria*; subtropical populations of this species are assumed to have lost a mechanism of reproductive diapause following secondary colonization of subtropical areas.
Overwintering strategies are diversified in the *obscura* species group: *D. alpina* and *D. subsilvestris* pass the winter in pupal diapause, *D. bifasciata* and *D. obscura* do so in reproductive diapause, and *D. subobscura* and *D. guanche* do so without entering diapause (see also Begon, 1976; Lumme & Lakovaara, 1983). This diversity may also suggest rapid radiation at an early phase of adaptations to temperate climates. It is not clear whether each type of diapause evolved once or repeatedly in this group. However, the distant phylogenetic relationship between the species having the same type of diapause suggests that repeated evolution is likely.

In *D. alpina*, the photoperiodic response was observed when pupae were maintained for more than 3 months at 15°C. However, this response would be concealed under natural conditions, because this species is univoltine in Shiga (the locality where the experimental strain originated) and usually pupates in mid to late August when daylength is not long (Beppu et al., 1996). The photoperiodic response of this species may be a remnant of past adaptations. At the early phase of adaptations of the *obscura* group to temperate climates, its distribution would be restricted to relatively warm areas and its members including the ancestor of *D. alpina* would have multivoltine life cycles and photoperiodically controlled diapause. In *D. alpina*, the photoperiodic response became to be concealed during the evolution of univoltine life cycle as a result of its adaptation to alpine and boreal climates. Ichijo, Beppu & Kimura (1992) also reported a concealed photoperiodic response in another univoltine species with reproductive diapause, *Drosophila moriakii*.

**Temperature tolerance**

Among the species studied, adult tolerance of cold is closely related to overwintering strategy and distribution. Adult flies of *D. obscura* and *D. bifasciata* that pass the winter in reproductive diapause displayed considerable tolerance of cold. The Yakutsk strain of *D. bifasciata* revealed more tolerance than the Sapporo strain. Adult *D. alpina* and *D. subsilvestris* with pupal diapause were less tolerant, while adult *D. subobscura* were moderately tolerant and therefore able to overwinter even in England or Germany. *D. guanche* occurring in the Canary Islands, where mild weather lasts throughout the year, was rather susceptible, although it is highly likely that this species has lost its tolerance over 20 years of laboratory rearing. It is not certain whether this species retains an ancestral characteristic (low tolerance), has lost tolerance consequent to its colonization of the Canary Islands or lost it since the 1970s in the laboratory. In the *melanogaster* species group, it has also been observed that tolerance is related to its distribution range (Kimura, 1988; Kimura et al., 1994; Ohtsu et al., 1998). Tolerance of cold would be a very important trait to survive the winter in temperate or arctic regions and subject to strong selection. However, in several species of the *melanogaster* species group, little geographic variation was observed in tolerance of cold (Kimura, 1982, 1988; Kimura et al., 1994). It is considered that genes controlling tolerance are highly coadapted or have serious pleiotropic-side effects in these species and that therefore tolerance does not change without drastic genetic reform, for example that leading to speciation.

Among the species studied, *D. alpina* and *D. subsilvestris* with pupal diapause had lower tolerance to high temperature in preimaginal development than the other species with reproductive diapause or without diapause. Tolerance to high temperatures may also have evolved in association with overwintering strategies.
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