Phylogeny of the *Drosophila obscura* group as inferred from one- and two-dimensional protein electrophoresis

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Abstract

The phylogenetic relationships of 15 species of the *obscura* group of *Drosophila* were analysed by use of one- and two-dimensional electrophoresis. Genetic distances based on two-dimensional data are five times smaller than those based on native proteins. From the data, it is proposed that the species radiation of the *obscura* group happened in two evolutionary bursts, the first one giving rise to at least four palearctic proto-lineages (*bifasciata, obscura* (including *D. subsilvestris*), *subobscura*, and *microlabis*) and one or two proto-nearctic lineages (*affinis, pseudoobscura*), and the second, more recent burst giving rise to the current speciation within lineages.

Key words: *Drosophila obscura* group – two-dimensional electrophoresis – one-dimensional electrophoresis – phylogenetic analysis

Introduction

The phylogenetic relationships among species of the *obscura* group of *Drosophila* have been intensively studied using traits of different complexity. On the basis of morphological characters, the group has been divided into two subgroups, *obscura* and *affinis*, both including nearctic as well as palearctic species (Sturtevant 1942; Buzzati-Traverso and Scossoirol 1955). The use of isozymes for tracing the evolution of the group has given rise to some discrepancies with respect to this traditional phylogeny. In a summary of their pioneering work, Lakovaara et al. (1976) divided the group into three distinct evolutionary branches, one lineage comprising the Eurasian *obscura* subgroup species, a second the American *affinis* subgroup species, and a third the American *obscura* subgroup species. Nevertheless, two species, *D. alpina* and *D. helvetica*, belonging morphologically to the *obscura* and the *affinis* subgroup, respectively, have not proven to be appreciably related to the other species. This group subdivision was supported by later enzymatic studies (Pinsker and Buruga 1982; Loukas et al. 1984). A fourth subgroup comprising the recently discovered new African species (Tsacas et al. 1985) has been added and termed the *microlabis* subgroup (Cariou et al. 1988). However, other authors, also using enzymatic traits, have obtained different phylogenies, mainly with respect to the *obscura* subgroup. Some palearctic species of this subgroup appear to be at least as distantly related to the other species of the *obscura* subgroup as to the nearctic species of the *pseudoobscura* subgroup (Marinković et al. 1977; Cabrera et al. 1983). Several new phylogenies of the group, obtained using mitochondrial-DNA-sequence (mtDNA) data have recently been proposed. The results can be summarized as follows: the traditional division of the group into two subgroups (*affinis* and *obscura*) does not correspond to the true phylogeny of the group (Barrio et al. 1992); *D. affinis* and *D. pseudoobscura* subgroups seem to be monophyletic groups with closer affinities to one another than to the palearctic *D. obscura* subgroup species (Beckenbach et al. 1993). The palearctic *obscura* subgroup seems to be a heterogeneous assemblage that could further be subdivided into several independent complexes (González et al. 1990; Barrio et al. 1994).

With the aim of reconciling the different phylogenies proposed, new molecular studies with representative species of all the subgroups hitherto described have been started. In this study, the phylogenetic relationships of 15 species were studied using one- and two-dimensional protein electrophoresis, a technique, that, by the inclusion of proteins with slower rates of evolutionary changes than the traditional allozymes, appears to be more useful for inferring the phylogenies of distantly related taxa (Spicer 1988).

Material and methods

Species

A total of 15 different *Drosophila obscura* group species were used in this study. Their geographic origin and sources are listed in Table 1.

Sample preparation

For two-dimensional electrophoresis (2DE), 20 mg adult male flies of each species, approximately 20 individuals each, were homogenized in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) with 8 M urea, 1% Nonidet P-40 detergent, 1% mercaptoethanol, and 5% Pharmacia ampholytes (2-D Pharmalyte). For one-dimensional native electrophoresis (1DNE), samples were simply homogenized in TE buffer. For one-dimensional sodium-dodecyl-sulfate (SDS) denatured electrophoresis (1DDE), samples were homogenized in TE buffer with 2.5% SDS and 5% mercaptoethanol, and heated at 100°C for 5 min. In all cases, homogenates were centrifuged at 300 000 × g for 15 min in a Beckman L7-55 ultracentrifuge and the supernatants kept at −70°C until use. On gels, 1 μl of each sample, at a concentration of 40 μg of wet fly weight/μl, was applied.

Electrophoresis

Electrophoresis were carried out using the PhastSystem equipment from Pharmacia LKB Biotechnology. Protocols and programmed conditions were, with minor modifications, those described in the PhastSystem owner’s manual. For 2DE, first-dimension isoelectric focusing was performed using PhastGel IEF 3–9 and the PhastGel sample applicator 8/1. After electrophoresis, gels were stained with Coomassie blue to locate the sample lanes, which were cut and used in the second dimension with PhastGel gradient 8–25, and PhastGel SDS buffer strips. For 1DNE, PhastGel gradient 8–25 and PhastGel native buffer strips were used. For 1DDE, PhastGel gradient 8–25 was also used but with PhastGel SDS buffer strips.
Table 1. Geographic origin of the 15 species used in this study

| Strains (abbreviations) | Geographic origin         | Laboratory source¹ |
|-------------------------|---------------------------|--------------------|
| **affinis subgroup**    |                           |                    |
| 1. D. affinis (aff)     | Nebraska, USA             | (1)               |
| 2. D. azteca (azi)      | Chilpancingo, Mexico      | (1)               |
| **obscura subgroup**    |                           |                    |
| (a) palearctic species  |                           |                    |
| 3. D. ambiguus (amb)    | Tübingen, Germany         | (2)               |
| 4. D. bifasciata (bif)  | Pavia, Italy              | (1)               |
| 5. D. guanche (gua)     | Bco. Inferno, Canary Islands | (3)            |
| 6. D. kitumenstis (kit)| Mt. Elgon, Kenya          | (1)               |
| 7. D. madeirensis (mad)| Faja da Nogueira, Madeira Island | (3) |
| 8. D. microlabis (mic)  | Mt. Elgon, Kenya          | (1)               |
| 9. D. obscura (observation) | Tübingen, Germany         | (2)               |
| 10. D. subobscura (sub) | Raices, Canary Islands    | (3)               |
| 11. D. subsilvestris (sus) | Tübingen, Germany        | (2)               |
| 12. D. tristis (tri)    | Tübingen, Germany         | (2)               |
| (b) nearctic species    |                           |                    |
| 13. D. miranda (mir)    | California, USA           | (5)               |
| 14. D. persimilis (per)| California, USA           | (2)               |
| 15. D. pseudoobscura    | California, USA           | (4)               |

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Detection
Gels were first Coomassie-blue stained and then silver stained following the protocol of the PhastGel-silver-kit instruction manual.

Data analysis
Patterns of bands or spots on the gels were compared between species by presence–absence criteria. Similarity was analysed using the Dice (1945) coefficient $S_D = 2a/(2a + u)$, where $a$ is the number of matches (bands or spots shared by the two species compared) and $u$ is the total number of mismatches (spots present in only one of these species). Following Spicer (1988), the logarithmic transformation of the similarity coefficient ($-\ln S_D$) was used as a measure of distance. Phylogenetic relationships were analysed assuming constant (UPGMA, Sneath and Sokal 1973) and variable (NJ, Saitou and Nei 1987) evolutionary rates, using the MEGA 1.01 program (Kumar et al. 1993).

Results
A mean of 125 ± 8.3 protein spots were reliably scored in the 2DE gels, 37.8 ± 4.1 bands was the mean for 1DNE gels, and 41.6 ± 1.0 bands was the mean for the 1DDE gels. An example of a rutiniary two-dimensional silver-stained gel is presented in Figure 1. Allelic differences among species are difficult to identify in two-dimensional electrophoresis (Avise 1983), therefore, spots were simply scored as either present or absent. Figure 2 shows located examples of the type of interspecific variation detected in 2DE gel comparisons. Matrices of genetic distances were calculated for 2DE (Table 2), 1DNE (Table 3) and 1DDE (Table 4) data sets. Mean distances obtained with 1DNE data (0.34 ± 0.08) were five times greater than those based on 2DE (0.07 ± 0.02) and 1DDE (0.06 ± 0.02). The phylgenetic relationships among species inferred from each set of data are shown in Figure 3 for 2DE, Figure 4 for 1DNE, and Figure 5 for 1DDE. Topologies with the same set of data but different evolutionary assumptions were practically identical: only D. bifasciata clustered differently in the NJ and UPGMA trees for 2DE (Fig. 3), but, in both cases, the split is very old. Phylogenetic trees based on different sets of data also showed similar relationships among species, only the relative clustering of the related triad, D.

Table 2. Matrix of distances ($-\log_a S_D$) based on two-dimensional electrophoretic data

| Species¹ | mad | gua | obs | amb | tri | sus | bif | mic | kit | pse | per | mir | aff | azt |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| sub      | 0.028 | 0.036 | 0.063 | 0.073 | 0.064 | 0.054 | 0.087 | 0.095 | 0.090 | 0.074 | 0.067 | 0.085 | 0.096 | 0.088 |
| mad      | 0.030 | 0.056 | 0.067 | 0.058 | 0.061 | 0.086 | 0.086 | 0.070 | 0.079 | 0.079 | 0.060 | 0.072 | 0.090 | 0.080 |
| gua      | 0.066 | 0.071 | 0.056 | 0.058 | 0.089 | 0.084 | 0.100 | 0.080 | 0.070 | 0.075 | 0.098 | 0.082 |       |     |
| obs      | 0.019 | 0.039 | 0.036 | 0.086 | 0.069 | 0.064 | 0.088 | 0.086 | 0.086 | 0.091 | 0.074 | 0.102 | 0.090 |     |
| amb      | 0.029 | 0.035 | 0.084 | 0.037 | 0.088 | 0.051 | 0.046 | 0.067 | 0.072 | 0.078 | 0.107 | 0.071 |     |     |
| sus      | 0.060 | 0.055 | 0.063 | 0.063 | 0.062 | 0.059 | 0.074 | 0.074 | 0.074 | 0.102 | 0.090 |     |     |     |
| bif      | 0.080 | 0.087 | 0.072 | 0.092 | 0.086 | 0.086 | 0.086 | 0.086 | 0.086 | 0.086 | 0.086 |     |     |     |
| mic      | 0.016 | 0.064 | 0.060 | 0.060 | 0.086 | 0.086 | 0.086 | 0.086 | 0.086 | 0.086 | 0.086 |     |     |     |
| kit      | 0.066 | 0.059 | 0.088 | 0.090 | 0.071 | 0.027 | 0.074 | 0.052 | 0.022 | 0.059 | 0.046 |     |     |     |
| pse      | 0.016 | 0.016 | 0.067 | 0.073 | 0.033 |       |     |     |     |     |     |     |     |     |
| per      | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.067 | 0.073 | 0.033 |     |     |     |     |     |     |
| mir      | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 |     |     |     |
| aff      | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 |     |     |     |
| azt      | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 |     |     |     |

¹ Abbreviations as in table 1
subobscura, D. madeirensis, and D. guanche was different: with 2DE, subobscura-madeirensis was the closest pair; with 1DNE, the closest pair was guanche-madeirensis; and with 1DDE, the order of the triad was not resolved at all (Figs 3, 4, 5). Discrepancies in the location of the branching points of the main clusters seem to indicate that all are very distinct and well-differentiated lineages. It seems that the radiation of the obscura group species had two important bursts, well separated in time. The older one gave rise to several branches, that, taking in account the species studied here, are actually represented by the bifasciata, pseudoobscura, affinis, microlabis, obscura (including sublicestris), and subobscura lineages. The second radiation gave rise to the extant species within these main lineages.

Discussion
The accumulation of new data and the addition of newly discovered species have changed the classical phylogenetic view of the D. obscura group, giving rise to a more congruent picture. It seems that the subdivision of the group into two subgroups, obscura and affinis, does not hold up anymore. All studies based on molecular data, beginning with the isozyme analysis by Lakoovara et al. (1972), as well as subsequent studies at the DNA level (Latorre et al. 1988; Goddard et al. 1990; Beckenbach et al. 1993), have demonstrated that the nearctic pseudoobscura lineage of the obscura subgroup is more related to the nearctic affinis subgroup than to the palearctic branch of its own subgroup. All clustering in this study, based on protein data, are also in accordance with this supposition. Chromosome homologies also support this assumption (Sturtevant and Novitski 1941; Lakoovara and Saura 1982). The D. obscura group subdivision into three subgroups (obscura, affinis and pseudoobscura), as proposed by Lakoovara et al. in 1972, is absolutely sound. Discrepancies in the monophyletic origin of the palearctic obscura subgroup began as early as 1974 when Farris, using the Lakoovara et al. (1972) data but applying the Wagner clustering procedure, considered D. bifasciata, a palearctic species, to be more closely related to the nearctic species of the pseudoobscura

| Species  | mad | gua | obs | amb | tri | sus | bif | mic | kit | pse | per | mir | aff | azt |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| sub      | 0.171 | 0.133 | 0.359 | 0.363 | 0.376 | 0.380 | 0.385 | 0.362 | 0.378 | 0.408 | 0.393 | 0.386 | 0.364 | 0.357 |
| mad      | 0.118 | 0.380 | 0.387 | 0.382 | 0.380 | 0.380 | 0.381 | 0.376 | 0.380 | 0.377 | 0.372 | 0.376 | 0.380 | 0.370 |
| gua      | 0.383 | 0.392 | 0.381 | 0.376 | 0.380 | 0.357 | 0.376 | 0.387 | 0.372 | 0.380 | 0.389 | 0.394 | 0.386 | 0.369 |
| obs      | 0.181 | 0.210 | 0.327 | 0.371 | 0.357 | 0.346 | 0.346 | 0.357 | 0.412 | 0.414 | 0.398 | 0.372 | 0.357 | 0.371 |
| amb      | 0.200 | 0.353 | 0.366 | 0.325 | 0.358 | 0.412 | 0.412 | 0.412 | 0.372 | 0.372 | 0.372 | 0.372 | 0.372 | 0.372 |
| tri      | 0.342 | 0.360 | 0.368 | 0.344 | 0.374 | 0.380 | 0.387 | 0.353 | 0.350 | 0.353 | 0.350 | 0.350 | 0.350 | 0.350 |
| sus      | 0.357 | 0.380 | 0.380 | 0.366 | 0.390 | 0.392 | 0.392 | 0.392 | 0.343 | 0.343 | 0.345 | 0.345 | 0.345 | 0.345 |
| bif      | 0.359 | 0.347 | 0.369 | 0.369 | 0.383 | 0.395 | 0.395 | 0.395 | 0.338 | 0.338 | 0.338 | 0.338 | 0.338 | 0.338 |
| mic      | 0.039 | 0.336 | 0.340 | 0.359 | 0.359 | 0.359 | 0.359 | 0.359 | 0.332 | 0.332 | 0.332 | 0.332 | 0.332 | 0.332 |
| kit      | 0.342 | 0.351 | 0.343 | 0.340 | 0.340 | 0.340 | 0.340 | 0.340 | 0.351 | 0.351 | 0.351 | 0.351 | 0.351 | 0.351 |
| pse      | 0.063 | 0.168 | 0.311 | 0.329 | 0.078 | 0.342 | 0.342 | 0.342 | 0.330 | 0.335 | 0.335 | 0.335 | 0.335 | 0.335 |
| per      | 0.342 | 0.342 | 0.342 | 0.342 | 0.342 | 0.342 | 0.342 | 0.342 | 0.330 | 0.335 | 0.335 | 0.335 | 0.335 | 0.335 |
| mir      | 0.332 | 0.332 | 0.332 | 0.332 | 0.332 | 0.332 | 0.332 | 0.332 | 0.332 | 0.332 | 0.332 | 0.332 | 0.332 | 0.332 |
| aff      | 0.187 | 0.187 | 0.187 | 0.187 | 0.187 | 0.187 | 0.187 | 0.187 | 0.187 | 0.187 | 0.187 | 0.187 | 0.187 | 0.187 |

Table 3. Matrix of distances based on native-protein electrophoretic data

1 Abbreviations as in Table 1
and the others to the different European clusters (Cabrera et al. 1983). This polyphyletic origin of the palearctic D. obscura subgroup representatives has been repeatedly confirmed by numerous studies of DNA data (Latorre et al. 1988; González et al. 1990; Goddard et al. 1990; Barrio et al. 1992; Marfany and González-Duarte 1993; Ruttkay et al. 1992; Beckenbach et al. 1993; Barrio et al. 1994). Isozyme analyses of recently discovered and rediscovered Afrotropical species of the group

Table 4. Matrix of distances based on SDS denatured protein electrophoretic data

| Species | mad | gua | obs | amb | tri | sus | bif | mic | kit | pse | per | mir | aff | azt |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| sub     | 0.012 | 0.012 | 0.063 | 0.065 | 0.079 | 0.063 | 0.046 | 0.089 | 0.089 | 0.049 | 0.049 | 0.063 | 0.063 | 0.088 |
| mad     | 0.012 | 0.063 | 0.065 | 0.075 | 0.062 | 0.046 | 0.088 | 0.088 | 0.048 | 0.048 | 0.063 | 0.063 | 0.063 | 0.088 |
| gua     | 0.063 | 0.065 | 0.065 | 0.062 | 0.046 | 0.088 | 0.088 | 0.048 | 0.048 | 0.063 | 0.063 | 0.063 | 0.063 | 0.088 |
| obs     | 0.016 | 0.038 | 0.038 | 0.078 | 0.076 | 0.065 | 0.065 | 0.052 | 0.052 | 0.052 | 0.052 | 0.052 | 0.052 | 0.052 |
| amb     | 0.038 | 0.046 | 0.065 | 0.088 | 0.088 | 0.088 | 0.088 | 0.052 | 0.048 | 0.048 | 0.048 | 0.048 | 0.048 | 0.048 |
| tri     | 0.059 | 0.100 | 0.088 | 0.079 | 0.079 | 0.063 | 0.063 | 0.063 | 0.063 | 0.063 | 0.063 | 0.063 | 0.063 | 0.063 |
| sus     | 0.052 | 0.076 | 0.076 | 0.052 | 0.052 | 0.076 | 0.076 | 0.062 | 0.062 | 0.062 | 0.062 | 0.062 | 0.062 | 0.062 |
| bif     | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| mic     | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| kit     | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| pse     | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| per     | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| mir     | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| aff     | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

1 Abbreviations as in Table 1

subgroup than to the other palearctic species of the subgroup. The same situation, also using isozyme data, was later found for the palearctic species D. subobscura (Marinković et al. 1978), D. ambigua (Cabrera et al. 1983), and D. obscura (Cariou et al. 1988). These findings led to the formulation of a new phylogenetic hypothesis for the species of the obscura group: a common ancestor gave rise to various lines in the palearctic region, one of which led to the American species

![Fig. 3. Phylogenetic relationships among species based on two dimensional electrophoretic data. a. UPGMA; b. NJ trees](image-url)
have also demonstrated a significant distance to the palearctic lineages (Cariou et al. 1988). The same has been found using chromosomes (Brehm et al. 1991), nuclear and mitochondrial ribosomal RNA sequences (Ruttikay et al. 1992), and cytb mtDNA sequences (T. Acosta et al. unpubl. data) as traits. A new separate subgroup (microlabis) has consequently been proposed for these African species (Cariou et al. 1988). The results of this study, based on protein data, are in total agreement with both the polyphyletic origin of the palearctic lineages of the obscura subgroup and the independent subgroup of the African species (Figs 3, 4, 5). However, none of the data presented here clarify the chronology of the speciation events leading to the major lineages proposed. In this respect, these data only support the incorporation of D. subsilvestris within the obscura lineage, as Lakoovara et al. (1972) already showed, and as the majority of studies carried out at DNA level have confirmed (e.g. González et al. 1990; Barrio et al. 1994). The phylogeny obtained with 1DDE data (Fig. 5), bringing the African species closer to the common ancestor of the group, though in accordance with one of the phylogenies proposed by Ruttikay et al. (1992), was not confirmed by either 2DE (Fig. 3) or 1DNE (Fig. 4) data, whose topologies are more in agreement with those proposed by Cariou et al. (1988) and Brehm et al. (1991). Finally, D. bifasciata appeared as an independent lineage that is not closely related to any other species, a result that is also in agreement with the majority of the phylogenies constructed where this species is included. It seems that, for the resolution of the hierarchy in this early radiation of the group, analyses of slower-evolving sequences and different sets of out-group species should be used.

With regard to the phylogenetic relationships among related species, it is evident that representatives of the affinis, pseudoobscura and subobscura subgroups are all monophyletic clusters. The topology of the triad pseudoobscura—persimilis—miranda with the first two species closer to each other (Figs 3, 4, 5) is in agreement with all previous analyses of this subgroup (Lakoovara et al. 1972; Goddard et al. 1990; Beckenbach et al. 1993; Barrio et al. 1992, 1994). With respect to the species triad obscura—ambigua—tristis, the data in this study favour the obscura—ambigua pair as being more related (Figs 3, 4, 5). This disagrees with the first studies based on electrophoretic data (Lakoovara et al. 1972, 1976; Pinsker and Buruga 1981) that considered obscura—tristis to be the closest pair. It is, however, in accordance with the majority of the more recent phylogenies obtained from very different sources of data such as chromosomal histone-gene localization (Felger and Pinsker 1987), specific satellite-DNA evolution (Bach-
Fig. 5. Phylogenetic relationships among species based on SDS denatured protein electrophoretic data. a. UPGMA; b. NJ Trees

Subobscura
Madeirensis
Guanche
Bifasciata
Obscura
Ambigua
Tristis
Subsilvestris
Pseudoobscura
Persimilis
Miranda
Affinis
Azteca
Microlabis
Kikutensis

Subobscura
Madeirensis
Guanche
Bifasciata
Obscura
Ambigua
Tristis
Subsilvestris
Pseudoobscura
Persimilis
Miranda
Affinis
Azteca
Microlabis
Kikutensis

mann and Sperlich 1993), mtDNA restriction-site maps (González et al. 1990), and mtDNA sequences (Barrio et al. 1994), as well as with unpublished results (T. Acosta et al.) on cytb sequences. The relative position of the three species in the subobscura cluster is more controversial. With the 2DE data, the species subobscura-madeirensis form a cluster (Fig. 3); with the 1DNE data, madeirensis-guanche is the most related pair (Fig. 4); and the 1DDE information fails to resolve the topology of the triad at all (Fig. 5). Phylogenies suggesting a closer relationship of D. madeirensis with D. subobscura, as obtained here with the 2DE set of data, were also obtained by Larruga and Pinsker (1984) and Loukas et al. (1984) from isozyme data. No discrepancies exist when chromosomal homologies (Krimbas and Loukas 1984; Moltó et al. 1987; Papaceit and Prevosti 1989; Brehm and Krimbas 1990a, b, 1992, 1993; Brehm et al. 1991), and analysis of hybrid sterility and developmental incompatibilities (Khadem and Krimbas 1991a, b, 1993; Papaceit et al. 1991) among these species are studied. The general agreement is that D. subobscura and D. madeirensis are the closer species pair. From the comparison of polytene chromosome patterns (Brehm and Krimbas 1992, 1993), D. guanche appeared to be the most ancestral species of this triad. The existence of a D. guanche specific-satellite DNA, not present in the genome of its sibling species D. subobscura and D. madeirensis (Bachmann et al. 1989), has been explained by considering the continental species D. subobscura as the parental species from which D. guanche and D. madeirensis originated through founder effects and geographic isolation. Using restriction-site maps of mtDNA, González et al. (1990) found, at a significant level, subobscura-madeirensis to be the closest pair of species. From four independent mtDNA sequences of these species, all but one analysis clustered madeirensis-subobscura (Barrio et al. 1994), and unpublished results (T. Acosta et al.) based on cytb sequences also favour the same pair relationship. Taking all this into account, the species D. madeirensis and D. subobscura should definitely be considered as being more related to each other than to D. guanche.

There are some hypothesis and time estimates for the evolution in the obscura group that are worth contrasting with the data presented here. In biogeographic analyses, Throckmorton (1975) proposed that the obscura group founder separated at the time of the temperate forest disjunction in mid-Miocene period, and that the final developments of present-day patterns were added by speciation events occurring within local regions during the Pliocene and Pleistocene ages that have continued into recent times. The hypothesis of two main radiation bursts is in good agreement with that proposition. If the calibration values for two-dimensional electrophoresis data (Spicer 1988: estimated at 118 million
years per unit of distance) is applied to the 2DE data in this study, the palaeartic proto-lineages of bifasciata, obscura, subobscura, microlabilis and one or two proto-arctic lineages (affinis–pseudoobscura) would have radiated about 9–10 million years ago in the middle-Miocene period. Applying the same criteria to the second and more recent burst, that gave rise to the speciation within these lineages, we obtain values in the range of 2–4 million years, which are in accordance with the geological events and fit well with time estimates given by other authors for these radiations (Lougas et al. 1984; Latorre et al. 1988; González et al. 1990; Barrio et al. 1992; Bachmann and Sperlich 1993; Beckenbach et al. 1993).

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Zusammenfassung
Phylogenie der Arten der Drosophila obscura-Gruppe abgeleitet von ein- und zweidimensionaler Protein-Elektrophorese

Die phylogenetischen Verwandtschaftsbeziehungen von 15 Arten der Drosophila-Gruppe der Gattung Drosophila wurden mit Hilfe von ein- und zweidimensionalen Elektrophorese von Proteinen untersucht. Die genetischen Distanzen, die aus den Ergebnissen der zweidimensionalen Elektrophoren ermittelt wurden, waren fünfmal kleiner als solche, die von nativen Proteinen kommen. Aufgrund der Untersuchungsergebnisse wird angenommen, daß die Radiation der Arten der obscura-Gruppe in zwei evolutiven Schüben erfolgt sei; der erste Schub hätte zu zumindest vier palaearktischen (bifasciata, obscura mit D. subtilisvistris, subobscura und microlabilis) und zwei proto-ne arktischen Linien (affinis, pseudoobscura) geführt. In einem zweiten Schub wären dann die end gültigen rezenten Arten entstanden.

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