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EVOLUTION OF THE OBSCURA GROUP DROSOPHILA SPECIES. II. PHYLOGENY OF TEN SPECIES BASED ON ELECTROPHORETIC DATA

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SUMMARY

Nine palearctic and one North American *Drosophila* species of the obscura group have been compared with respect to electrophoretically detectable differences at 24 enzyme loci. The genetic distances between the species have been calculated. The constructed tree is divided into two evolutionary lineages. The first comprises the species *D. pseudoobscura* and *D. helvetica* and the second the palearctic species of the *obscura* subgroup. The latter splits into two lineages. One lineage leads to *D. bifasciata* and *D. subsilvestris*; the other splits further to two sublineages: the sublineage of *D. obscura*, *D. tristis* and *D. ambigua* and the sublineage of *D. guanche*, *D. subobscura* and *D. madeirensis*.

1. INTRODUCTION

The *Drosophila* species of the obscura group can be further clustered into two subgroups (Lakovaara and Saura, 1982): *obscura* and *affinis*. The *obscura* subgroup comprises fourteen palearctic species: *D. obscura* Fallen, *D. ambigua* Pomini, *D. subobscura* Collin, *D. madeirensis* Monclús, *D. guanche* Monclús, *D. bifasciata* Pomini, *D. subsilvestris* Hardy and Kaneshiro, *D. tristis* Fallen, *D. eskoi* Lakovaara and Lankinen, *D. alpina* Burla, *D. imaii* Moriwaki and Okada, *D. eniwae* Takada, Beppu and Toda, *D. epiobscura* Parshad and Duggal and *D. tsukubaensis* Takamori and Okada (1983). Among the American species of the *obscura* subgroup, *D. pseudoobscura* Frolova is the most widespread one, while among the species of the *affinis* subgroup, *D. helvetica* Burla is the only palearctic species: all others, belonging to this subgroup, are widespread in North America.

In this study we have constructed a phylogenetic tree based on electrophoretic data concerning ten species of the obscura group, nine belonging to the *obscura* subgroup and one to the *affinis* subgroup (table 1). The phylogeny is based on 24 gene loci coding for enzymes. Our aim is (i) to compare this phylogeny to that proposed by Lakovaara *et al.* (1976), since eight out of the 21 loci studied by them were not studied by us, while eleven loci were exclusively studied by us, and (ii) to compare the phylogeny of *D. subobscura*, *D. madeirensis* and *D. guanche*, based on chromosomal gene arrangements (Krimbas and Loukas, this issue), to that obtained by electrophoretic data.

2. MATERIAL AND METHODS

(i) *Electrophoresis*

Horizontal starch gel electrophoresis was employed to study the following enzyme systems: 6-phosphogluconate dehydrogenase (6-*PGD*); malate

TABLE 1

The electrophoretic mobilities of the most common allozymes at 24 loci in 10 species of the obscura group

	Species									
Enzymes	ob	am	so	ma	gu	bi	ss	tr	ps	he
6-PGD	1·42	1·42	1·00	1·00	0·95	1·42	1·42	1·42	1·34	1·58
Mdh	1·37	2·53	1·00	1·00	1·00	2·53	2·00	2·21	1·68	3·37
α-GPD	1·00	1·00	1·00	1·00	1·00	1·37	1·00	1·00	1·00	1·00
Idh	0·77	0·77	1·00	1·10	1·00	0·61	0·61	0·77	1·17	1·17
Phi	1·08	1·08	1·00	1·08	1·00	1·25	1·25	1·08	1·00	1·25
Adh	1·00	1·00	−1·00	−1·00	−1.00	0·29	0·29	1·00	−1·11	−1·78
Odh	1·00	0·90	1·00	1·00	1·00	1·00	1·00	1·00	1·07	0·96
T0-1	3·55	1·00	1·00	1·00	1·00	1·00	1·00	3·55	S	S
T0-2	1·00	1·04	1·00	1·00	1·04	1·00	1·04	1·00	1·00	2·04
Hk-1	1·00	1·15	1·00	S	0·95	2·48	1·41	1·15	1·41	1·55
Hk-3	1·00	1·00	1·00	1·00	1·00	0·99	1·00	1·00	0·99	1·00
Pep-1	1·06	1·50	0·40	S	1·94	2·37	1·50	1·50	1·06	1·19
Pep-2	1·03	0·98	1·00	1·00	1·02	1·12	1·03	0·98	0·94	1·03
Pep-3	1·12	1·12	1·00	1·08	1·08	0·85	1·09	1·12	1·14	1·00
G-6-PD	1·07	0·87	1·00	1·00	1·15	1·15	1·15	0·87	1·15	0·87
Me	0·72	0·82	1·00	1·10	0·84	0·92	0·72	0·91	0·54	0·91
Pgm-1	1·06	1·06	1·00	1·00	1·00	2·09	0·63	1·00	0·94	0·63
Pgm-2	0·90	0·90	1·00	1·00	1·07	2·00	0·40	0·90	0·80	0·40
Diaph	1·06	1·06	1·00	1·06	1·06	0·94	1·06	1·06	1·06	1·06
Acph	0·33	2·50	1·00	1·00	1·00	3·00	2·50	0·33	1·67	0·75
Aph	1·42	1·68	1·00	1·00	0·68	1·61	1·89	1·79	1·42	1·42
Ao	1·30	1·00	1·00	1·07	1·07	1·04	1·04	1·17	1·27	1·00
Lap	0·72	0·90	1·00	1·02	1·07	1·00	0·80	0·96	0·94	1·02
Xdh	1·00	0·97	1·00	1·00	1·08	0·90	1·08	1·08	1·08	1·12

Abbreviations used: ob = *D. obscura*; am = *D. ambigua*; so = *D. subobscura*; ma = *D. madeirensis*; gu = *D. guanche*; bi = *D. bifasciata*; ss = *S. subsilvestris*; tr = *D. tristis*; ps = *D. pseudoobscura*; he = *D. helvetica*

dehydrogenase (*Mdh*); α-glycerophosphate dehydrogenase (*α-GPD*); isocitric dehydrogenase (*Idh*); phosphohexose-isomerase (*Phi*); alcohol dehydrogenase (*Adh*); octanol dehydrogenase (*Odh*); tetrazolium oxidase (two loci: *To*-1 and *To*-2); hexokinase (two loci: *Hk*-1 and *Hk*-3); peptidase (three loci: *Pep*-1, *Pep*-2 and *Pep*-3); glucose-6-phosphate dehydrogenase (*G-6-PD*); malic enzyme (*Me*); phosphoglucomutase (two loci: *Pgm*-1 and *Pgm*-2); diaphorase (*Diaph*); acid phosphatase (*Acph*); alkaline phosphatase (*Aph*); aldehyde oxidase (*Ao*); leucine aminopeptidase (Lap); and xanthine dehydrogenase (*Xdh*). Buffer solutions and staining methods are those given by Loukas and Krimbas (1980) except for the enzymes: *To* for which a TRIS–Boric acid–EDTA solution (0·18 M TRIS + 0·1 M boric acid + 0·004 M EDTA.2Na) was used as electrode buffer and a dilution 1 : 4 of this buffer for preparing the gels; *Idh* for which a TRIS-HCl solution (0·25 M TRIS, *p*H = 8·5) was used as electrode buffer and a dilution 1 : 4 of this buffer for preparing the gels; *Pep*-2 for which instead of L-leucyl-L-tyrosine the dipeptide L-phenylalanyl-L-proline was used as a substrate; and *Pgm*-2 for which a 0·041 M sodium citrate solution (*p*H = 7·5) was used as electrode buffer and a 0·005 M histidine solution (*p*H = 7·5) for preparing the gels.

The adult stage was used in all enzymes studied except for *Lap* (pupal stage) and *Aph* (third instar larvae).

For naming sites of activity, every allozyme band was identified by a number denoting the position of the band relative to the position of the band yielded by the most common allele of the species *D. subobscura*. Loci with similar *in vitro* enzymatic activity were distinguished by a number (1, 2 or 3) denoting their succession from the origin: the smaller number indicating less migration.

(ii) *The strains*

The data for *D. obscura* are based on twenty-nine isofemale strains from Mt Parnes (Greece), nine from Karpenissi (Greece); five from Switzerland; one from Vitebsk (U.S.S.R.); and one from the Spanish Pyrenees (Caralps). For *D. ambigua* two strains from Karpenissi (Greece); one from Mt Parnes (Greece); two from Alma Ata (U.S.S.R.); and one from Switzerland were used. For *D. subobscura* we used isogenic strains kept in the Department of Genetics, Agricultural College of Athens, which carried the most common alleles of all the loci studied throughout the whole species distribution. We also analysed one strain of *D. madeirensis* and one strain of *D. guanche* (kindly provided by Professor Antonio Prevosti); two strains of *D. bifasciata*, one from Switzerland and one from Pavia (Italy); three strains of *D. subsilvestris*, two from Switzerland and one from Karpenissi (Greece); three strains of *D. tristis* from Switzerland; one strain of *D. pseudoobscura* from U.S.A.; and finally two strains of *D. helvetica* from Switzerland. Flies of all the available strains of each species were electrophoresed for each gene to establish the most common allele of the particular locus. Then, strains of all ten species, bearing the common alleles, were run side-by-side for the interspecies comparison.

3. Results

(i) *Genetic polymorphism*

In table 1 are given the most common alleles of each species for all 24 loci. The negative sign in front of some alleles of the *Adh* locus indicates a cathodal migration. The capital letter S stands for a silent allele since no reaction was repeatedly observed in the gels.

The following twenty loci displayed very little or no intraspecies variation: 6-*PGD*—This enzyme yielded five different electrophoretic phenotypes. One strain of *D. obscura* and one of *D. ambigua* were found polymorphic. *Mdh*—The ten *Drosophila* species belong to seven different electrophoretic phenotypes. Two strains of *D. obscura* and two of *D. ambigua* were found to be polymorphic. (In this study a polymorphic strain for a particular locus always beats two alleles, one of which is usually the common one.) *α-GPD*—This yielded two different electrophoretic phenotypes. Only one strain of *D. obscura* was found to be polymorphic. *Idh*—This enzyme classified the ten species into five electrophoretic phenotypes. Five strains of *D. obscura* displayed polymorphism for this locus. *Phi*—No intraspecies electrophoretic variation was detected. This enzyme yielded three different electrophoretic phenotypes. *Adh*—The ten species belong to five electrophoretic phenotypes. Ten strains of *D. obscura*

were found to be polymorphic. *Odh*—No intraspecies electrophoretic variation was observed. The interspecies comparison revealed four different electrophoretic classes. *To*-1 and *To*-2—No polymorphic strains were found for these two loci except for one *D. ambigua* strain for *To*-1. Both loci yielded three different electrophoretic phenotype. *Hk*-1—Although *Hk*-1 was invariant within species, among species it showed considerable variation. The ten species were classified into seven electrophoretic phenotypes. *Hk*-3—This yielded two electrophoretic classes. Intraspecies variation was detected only in *D. obscura*. Thus thirteen out of forty-five strains of *D. obscura* displayed two alleles, one of which was the common one. *Pep*-1—This classified the ten species into seven electrophoretic phenotypes. Intraspecies polymorphism was detected in six strains of *D. obscura* and three of *D. ambigua*. *Pep*-2—No intraspecies variation was detected except for three *D. obscura* and for one *D. subsilvestris* strains. The interspecies comparison revealed six electrophoretic phenotypes. *Pep*-3—This also yielded six electrophoretic phenotypes. All the strains except for one *D. ambigua* strain were monomorphic. *G-6-PD*—Four strains of *D. obscura* and one of *D. ambigua* displayed polymorphism for *G-6-PD*. This locus grouped the ten species into four electrophoretic classes. *Me*—This classified the ten species into eight different electrophoretic phenotypes. No intraspecies variation was detected except for one *D. bifasciata* strain. *Pgm*-1—This yielded five electrophoretic phenotypes. Six strains of *D. obscura* and one of *D. bifasciata* were found polymorphic. *Pgm*-2—This displayed six different electrophoretic phenotypes. No intraspecies variation was detected. *Diaph*—The ten species were classified into three electrophoretic classes. No intraspecies variation was detected. *Acph*—The interspecies comparison revealed six electrophoretic phenotypes. Twelve strains of *D. obscura*, one of *D. tristis* and one of *D. bifasciata* were found to be polymorphic.

Finally, the following four loci displayed moderate or high intraspecies variation: *Aph*—This classified the ten species into seven distinguishable electrophoretic phenotypes. Sixteen strains of *D. obscura*, one of *D. subsilvestris*, one of *D. tristis* and one of *D. ambigua* were found to be polymorphic. *Ao*—This yielded six different electrophoretic phenotypes. Twenty strains of *D. obscura*, three of *D. ambigua*, one of *D. bifasciata*, one of *D. subsilvestris* and two of *D. tristis* proved to be polymorphic. *Lap*—This classified the ten species into eight electrophoretic phenotypes. Seventeen strains of *D. obscura*, one of *D. ambigua* and one of *D. bifasciata* were found polymorphic. *Xdh*—The ten species belong to five electrophoretic phenotypes. Eleven strains of *D. obscura*, three of *D. ambigua*, one of *D. bifasciata*, one of *D. subsilvestris* and one of *D. tristis* were found to be polymorphic.

All the above remarks on the intraspecies genetic polymorphism do not concern *D. subobscura*, which has already been described by Loukas *et al.* (1979).

(ii) *Phylogeny*

Above the diagonal of table 2 we give the index of electrophoretic identity between the species X and Y, I_{XY}, which (in our case) is defined as the fraction of loci at which species X and Y share the same common allele. The corresponding genetic distances, D_{XY}, with standard errors, $S_{D_{XY}}$, are indicated below the diagonal (Nei, 1971). From the distance matrix

TABLE 2

Estimates of I (*above the diagonal*), D *and* S_D (*below the diagonal*) *between all possible pairs of 10 species of the obscura group. Further explanation in the text*

	ob	ab	so	ma	gu	bi	ss	tr	ps	he
D. obscura	—	0·417	0·250	0·292	0·167	0·125	0·292	0·542	0·208	0·208
D. ambigua	0·875 ±0·241	—	0·167	0·208	0·208	0·125	0·333	0·542	0·083	0·208
D. subobscura	1·386 ±0·354	1·790 ±0·456	—	0·625	0·417	0·167	0·167	0·208	0·125	0·167
D. madeirensis	1·231 ±0.318	1·570 ±0·398	0·470 ±0·158	—	0·458	0·125	0·208	0·292	0·125	0·167
D. guanche	1·790 ±0·456	1·570 ±0·398	0·875 ±0·241	0·781 ±0·222	—	0·125	0·333	0·250	0·208	0·125
D. bifasciata	2·079 ±0·540	2·079 ±0·540	1·790 ±0·456	2·079 ±0·540	2·079 ±0·540	—	0·333	0·125	0·125	0·042
D. subsilvestris	1·231 ±0·0318	1·100 ±0·289	1·790 ±0·456	1·570 ±0·398	1·100 ±0·289	1·100 ±0·289	—	0·292	0·208	0·292
D. tristis	0·612 ±0·188	0·612 ±0·188	1·570 ±0.398	1·231 ±0·318	1·386 ±0·354	2·079 ±0·540	1·231 ±0·318	—	0·167	0·208
D. pseudoobscura	1·570 ±0·398	2·489 ±0·678	2·079 ±0·540	2·079 ±0·540	1·570 ±0·398	2·079 ±0·540	1·570 ±0·398	1·790 ±0·456	—	0·208
D. helvetica	1·570 ±0·398	1·570 ±0·398	1·790 ±0·456	1·790 ±0·456	2·079 ±0·540	3·170 ±0·975	1·231 ±0·318	1·570 ±0·398	1·570 ±0·398	—

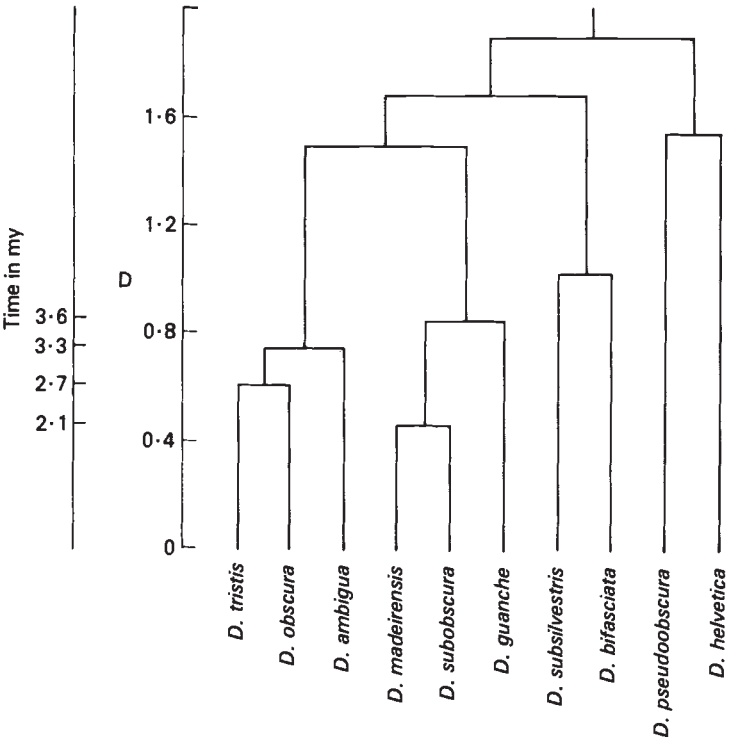

FIG. 1. A phylogenetic tree of ten *Drosophila* species of the obscura group. The time scale (in million years) is calculated as the mean of the two estimates indicated in the text.

of this table the phylogenetic tree of fig. 1 was constructed, according to the Unweighted Pair Group Mean Analysis (UPGMA) method of Sneath and Sokal (1973).

4. Discussion

From the electrophoretic data it follows that the gene *Me* and, to a lesser degree, the genes *Mdh*, *Hk*-1 and *Pep*-1 can be used as genetic markers to distinguish most of the species belonging to the obscura group, not only because of the great number of the interspecies electrophoretic phenotypes which they yield but also because of their low degree of intraspecies genetic variation. It is the latter requirement which excludes *Lap* as a good genetic marker. It must be noted that the simultaneous study of *Pep*-1 and *Pep*-3 could distinguish all species studied. (These two loci are stained on the same slab by the same substrate).

Lakovaara *et al.* (1976) in their latest paper on the phylogeny of the obscura group, which included nine of the ten species studied here, utilised 15 enzymatic systems corresponding to 21 loci, of which three were invariable between species.

Cabrera *et al.* (1983) utilised 30 enzyme systems corresponding to 67 loci to study the electrophoretic relationships among six species of the obscura group, five of which were in common with the present study. Our

study includes 19 enzyme systems corresponding to 24 variable (between species) loci. In table 3 we indicate, for each locus, the mobility of the most common allozyme band of each species, starting from the fastest migrating band (at left) towards the slowest migrating one (to the right). Species which yielded allozymes with different mobilities are separated by semicolons, while a dash between two species denotes identical mobility of the corresponding allozymes. The data of Lakovaara *et al.* (1976) and Cabrera *et al.* (1983) presented here are restricted to the species in common with the present study: nine species for the first study [except for *Acph*, for which only seven species were studied (Lakovaara *et al.*, 1972) and *Ao* and *Xdh*, for which only eight species were studied (Lakovaara *et al.*, 1976)], and five species for the second study.

The differences observed in the electrophoretic data among these three studies may be attributed:

(a) To the wrong choice of the common allele in each particular case. This is probably due to the small number of strains available for some of the species in each study. We tried to overcome this difficulty partially by using, for some of the species studied, strains from different localities. This difficulty is also partially overcome by the fact that the majority of the loci studied retain no or very little intraspecies variation.

(b) To the utilization of electrophoretic techniques with very little discrimination efficiency. This is obvious from the very small differences in migration of the allozymes reported by Lakovaara *et al.* (1976) in comparison to those of the present study. The same cause could also explain the differences in electrophoretic phenotypes, between the present study and that of Cabrera *et al.* (1983) as far as the species *D. subobscura*, *D. madeirensis* and *D. guanche* are concerned. It could be argued that these differences are due to the fact that we used only one strain for *D. madeirensis* and one for *D. guanche* while Cabrera *et al.* (1983) used samples from natural populations. We must, however, not overlook the fact that there is very little variation within *D. guanche* and *D. madeirensis*, when the same 22 genes (common in both studies) are considered (Cabrera *et al.*, 1983). It is, therefore, difficult to admit that a single unselected strain showed homozygosity for a rare allele. Thus it seems that the greater number of identical bands detected by Cabrera *et al.* (1983), in all pairwise comparisons between the three species in question, is mainly due to electrophoretic techniques not permitting perfect discrimination. It must also be noticed that our strain of *D. madeirensis* is homozygous for a silent allele for *Pep*-1, the existence of which was not reported by Cabrera *et al.* (1983).

Pinsker and Buruga (1982) detected very little genetic variation in population samples of the species *D. obscura*, *D. ambigua*, *D. subobscura*. *D. subsilvestris*, *D. tristis* and *D. helvetica* for the loci *Adh*, *α-GPD*, *Idh*, *Mdh*, *Me*, *Odh*, 6-*PGD* and *Pgm*. However, their data are not included in table 3 since, except for a small difference concerning the electrophoretic phenotypes of the species *D. subsilvestris* and *D. helvetica* for the *Pgm* locus, all their electrophoretic data were identical to those of the present study.

TABLE 3

Electrophoretic mobilities of the common alleles for 22 loci. The data of Lakovaara et al. *(1976) and Cabrera* et al., *(1983) concern only the common species with the present study. Further explanations in the text*

Loci	Present study	Lakovaara *et al.* (1976, 1972)	Cabrera *et al.* (1983)
6-*PGD*	he; ob-ab-bi-ss-tr; ps; so-ma; gu	—	am: so-ma-gu-ps
Mdh	he; am-bi; tr; ss; ps; ob; so-ma-gu	he; am; bi; tr; ss; ps; ob; gu; so	am; ps; so-ma-gu
α-GPD	bi; ob-am-so-ma-gu-ss-tr-ps-he	bi; ob-am-so-gu-ss-tr-ps-he	am-so-ma-gu-ps
Idh	ps-he; ma; so-gu; ob-am-tr; bi-ss	ps-he; so-gu; ob-am-tr; ss; bi	ps; ma; so-gu; am
Phi	bi-ss-he; ob-am-ma-tr; so-gu-ps	—	am-so-ma-gu-ps
Adh	ob-am-tr; bi-ss; so-ma-gu; ps; he	ob-am-tr; bi-ss; so-gu; ps; he	am; so-ma-gu; ps
Odh	ps; ob-so-ma-gu-bi-ss-tr; he; am	ps; am-so-gu-bi-ss-tr; he; am	am; ps; so-ma-gu
Hk-1	bi; he; ss-ps; am-tr; ob-so; gu; ma*	he; bi; ps; ss; tr; am; ob-gu; so	ps; am-so; gu; ma
Hk-3	ob-am-so-ma-gu-ss-tr-he; bi-ps	ob-am-tr; he; so-gu-ss; bi; ps	ps; am-so-ma-gu
Pep-1	bi; gu; am-ss-tr; he; ob-ps; so; ma*	—	gu; am; ps; so; ma
Pep-2	bi; ob-ss-he; gu; so-ma; am-tr; ps	—	am-so-ma-gu; ps
Pep-3	ps; ob-am-tr; ss; ma-gu; so-he; bi	—	am-ps; ma-gu; so
G-6-PD	gu-bi-ss-ps; ob; so-ma; am-tr-he	—	am-so-ma-gu-ps
Me	ma; so; bi; tr-he; gu; am; ob-ss; ps	so; bi; gu-tr; he; am; ob-ss; ps	ma; so; gu; am; ps
Pgm-1	bi; ob-am; so-ma-gu-tr; ps; ss-he	bi; so-gu; am; ob-tr; ps; ss; he	so-ma-gu; am; ps
Pgm-2	bi; gu; so-ma; ob-am-tr; ps; ss-he	—	ps; ma; so-gu; am
Diaph	ob-am-ma-gu-ss-tr-ps-he; so; bi	—	am; ps; gu; ma; so
Acph	bi; am-ss; ps; so-ma-gu; he; ob-tr	bi; tr; ps; ss; ob; am; so	am-ps; so-ma-gu
Aph	ss; tr; am; bi; ob-ps-he; so-ma; gu	tr; am-ss; bi-ps; ob-gu-he; so	am; ps; so-ma; gu
Ao	ob; ps; tr; ma-gu; bi-ss; am-so-he	ob; ps; am-he; bi-ss-tr; so	ps; am-so-ma-gu
Lap	gu; ma-he; so-bi; tr; ps; am; ss; ob	ps; he; so-tr; am-gu; ob-bi; ss	so-ma; am-ps; gu
Xdh	he; gu-ss-tr-ps; ob-so-ma; am; bi	he; tr-ps; ob-am-ss; so; bi	ps; gu; so; ma; am

* Homozygous for a silent allele.

The most significant features of the phylogeny of fig. 1 are the following:

(i) There is a clear cut separation of the eight palearctic species of the *obscura* subgroup from the species *D. helvetica* and *D. pseudoobscura.*

(ii) The lineage leading to the *obscura* subgroup splits into two lineages. One lineage leads to *D. bifasciata* and *D. subsilvestris*; the other splits further into two sublineages: the sublineage of *D. obscura, D. tristis* and *D. ambigua* and the sublineage of *D. guanche, D. subobscura* and *D. madeirensis.* It is very important to note that, in this last sublineage, *D. subobscura* and *D. madeirensis* cluster together.

Thus, *D. subobscura, D. madeirensis* and *D. guanche* are apparently closely related species, a finding which agrees with the results of Krimbas and Loukas (in this issue). These authors examined the gene arrangements using the giant chromosomes of these three species and those of their F_1 hybrids. They found that *D. subobscura* is cytologically closer to *D. madeirensis* than to *D. guanche*, while *D. guanche* is closer to *D. madeirensis.* The comparison of *D. guanche* with *D. madeirensis* showed that there are 4 inversions unique to *D. guanche* and 2 inversion shared only by these two species (O_3 and a small distal inversion involving the section 16 BCD of the A chromosome). On the other hand, the comparison of *D. subobscura* with *D. madeirensis* showed that these two species differ only by the two inversions which *D. madeirensis* shares with *D. guanche.* These findings also agree with the results obtained with interspecies crosses. Thus the only possible crosses between the three species in question were: ♀ *D. madeirensis* × ♂ *D. guanche* and ♀ *D. madeirensis* × ♂ *D. subobscura.* The first cross yielded a small number of F_1 sterile females and males and the second sterile F_1 males but fertile F_1 females which can be backcrossed to both parents.

The electrophoretic data presented in this paper, as far as the species *D. subobscura, D. madeirensis* and *D. guanche* are considered, do not support the conclusion of Cabrera *et al.* (1983), that *D. madeirensis* is more closely related to *D. guanche* than to *D. subobscura.* On the contrary, our data suggest a closer relation of *D. madeirensis* and *D. subobscura* which both are more apart from *D. guanche.* Although many more electrophoretic data, than those presented both by Cabrera *et al.* (1983) and the present study, would be needed to get an absolute certainty on the exact topography of the phylogenetic tree, it is significant to note that data based on chromosomal gene arrangements and on the possibility of obtaining interspecific hybrids are in absolute concordance with the electrophoretic data of the present study. This is a further suggestion on the correctness of the phylogenetic topology presented here.

Using the data presented by Lakovaara *et al.* (1976), concerning the nine species, common to both studies, we constructed a phylogenetic tree: this tree was found to be quite similar in topology to the tree depicted in fig. 1. Its only difference concerns the species *D. subsilvestris* which was found to be in the same group with *D. ambigua, D. tristis* and *D. obscura* (there is yet another branching, leading to *D. subsilvestris*, in the lineage of this group before this lineage gave rise to the three species in question). These matching results are due not only to the moderate similarity of the electrophoretic data reported in both studies, but also to the fact that usually

the unique phenotypes displayed by a species for a particular locus differ in the two studies and the method applied recognises only identities, treating the same way differences in electrophoretic phenotypes. Thus the different unique phenotypes between the two studies (for the same species and locus) have exactly the same influence on the estimation of the genetic distances.

Marinkovic *et al.* (1977) constructed a phylogenetic tree of five species of the obscura group based on allelic frequencies of six loci only. They suggested a phylogeny in which *D. subobscura* is genetically more similar to the North American species *D. pseudo-obscura* and *D. persimilis* than to the two European species *D. obscura* and *D. bifasciata.* Also, Cabrera *et al.* (1983) found that *D. ambigua* is more closely related to American species *D. pseudoobscura* than to the cluster of species from the old world. (These two species can eventually hybridise). On the contrary, our results are in close agreement with those reported by Lakovaara *et al.* (1976): the palearctic species of the *obscura* subgroup cluster together and differ from the American species.

The phylogenetic tree can also be viewed in an absolute time scale. It is possible to get approximate time divergence estimations in million years from the genetic distances. For this purpose we intend to use a method employed in vertebrate studies in spite of the fact that it is not quite clear whether this calibration applies also to insects. Thus, when D is smaller than 0·2 the time in years is very roughly equal to $D \times 5 \times 10^6$, when one fourth of the amino acid substitutions is electrophoretically detected, or $D \times 3{\cdot}75 \times 10^6$ when one such third is distinguished (Nei and Roychoudhury, 1982). In this study, only one buffer system was used for every enzyme locus, but the most adequate for it. Since a very tedious amount of repeated electrophoreses, in all kinds of species combinations, was performed in order to get direct comparison between every possible pair of species running one next to the other, and since repeated electrophoreses were performed with different durations of time when a suspected small difference in mobility was observed, we feel that in our case the multiplication factor lies somewhere between these two values. D, however, is in all cases higher than 0·2. Thus the times noted in the scale of fig. 1 should be taken only as indications of orders of magnitude. It seems that *D. subobscura* and *D. madeirensis*, the pair of most closely related species studied by us, diverged more than 2 million years B.P.

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