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Phylogeny of the *Drosophila obscura* Species Group Deduced from Mitochondrial DNA Sequences

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Abstract. Approximately 2 kb corresponding to different regions of the mtDNA of 14 different species of the *obscura* group of *Drosophila* have been sequenced. In spite of the uncertainties arising in the phylogenetic reconstruction due to a restrictive selection toward a high mtDNA A+T content, all the phylogenetic analysis carried out clearly indicate that the *obscura* group is formed by, at least, four well-defined lineages that would have appeared as the consequence of a rapid phyletic radiation. Two of the lineages correspond to monophyletic subgroups (i.e., *affinis* and *pseudoobscura*), whereas the *obscura* subgroup remains heterogeneous assemblage that could be reasonably subdivided into at least two complexes (i.e., *subobscura* and *obscura*).

Key words: Mitochondrial DNA — Nucleotide sequences — *Drosophila* — Rapid phyletic radiation — Molecular phylogeny

Introduction

The study of several species of the *Drosophila obscura* group during the last decades has contributed very much to the development of the evolutionary genetics (Lakovaara and Saura 1982). Of them, the American species *D. pseudoobscura* (Dobzhansky and Powell 1975) and the European species *D. subobscura* (Krimbas and Loukas 1980) have been favorite subjects of numerous evolutionary, ecological, and behavioral studies.

Classically the *obscura* group was subdivided into the *D. obscura* subgroup (Nearctic and Palearctic species) and the *D. affinis* subgroup (almost exclusively Nearctic species) according to morphological considerations (Sturtevant 1942; Buzzati-Traverso and Scossiroli 1955). Throughout the 1970s allozyme characters provided useful information for phylogenetic analysis of the *D. obscura* group (Lakovaara et al. 1972, 1976; Marinkovic et al. 1978; Lakovaara and Keränen 1980; Pinsker and Buruga 1982; Cabrera et al. 1983; Loukas et al. 1984). Lakovaara and Saura (1982) proposed the division of the group into three subgroups: the *D. obscura* subgroup, the *D. pseudoobscura* subgroup, and the previously described *D. affinis* subgroup. Mitochondrial DNA (mtDNA) restriction analyses (Latorre et al. 1988; González et al. 1990; Barrio et al. 1992) and DNA-DNA hybridization of single-copy nuclear DNA (Goddard et al. 1990) supported the existence of the new *D. pseudoobscura* subgroup and pointed out the heterogeneity of the *D. obscura* group.

African species of the group were discovered only few years ago (Tsacas et al. 1985), and as a result of their allozymic analysis (Cariou et al. 1988) a fourth subgroup was proposed, the *microlabis*. Nuclear and mitochondrial rRNA sequence analyses (Ruttkay et al. 1992) and chromosomal studies (Brehm and Krimbas 1990, 1992, 1993; Brehm et al. 1991) also give support to the existence of

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such subgroup. However, in spite all these studies, the monophyletic character of some of these subgroups and the phylogenetic relationships between and within subgroups have been questioned and still remain elusive (Goddard et al. 1990; González et al. 1990; Barrio et al. 1992).

An important recent contribution includes the extensive survey of the phylogenetic relationships of the *D. obscura* group inferred from mitochondrial cytochrome c oxidase subunit II (COII) sequences carried out by Beckenbach et al. (1993). They concluded that *D. affinis* and *D. pseudoobscura* subgroups are monophyletic groupings that have closer affinities to one another than they have to the *D. obscura* subgroup. Nevertheless, as they suggested, the final resolution of the relationships between the endemic North American species and the members of the *D. obscura* subgroup will probably require analysis of more members of the latter subgroup and more genes.

In the present study we analyze the phylogenetic relationships of the *D. obscura* group species, including representatives of the *D. affinis* and *D. pseudoobscura* subgroups and all the available species from the *D. obscura* subgroup, on the basis of partial sequences of four mitochondrial genes (lrRNA, cyt b, ND1, and ND5) and two trRNAs.

Materials and Methods

Drosophila species. Partial mtDNA sequences were obtained for 14 species of the *D. obscura* group. Three species belong to the *D. affinis* subgroup: *D. affinis* from Crystal Lake (Nebraska, obtained from the *Drosophila* collection at Bowling Green, OH, stock number 14012-0141.0). *D. algonquin* from Honeoye Falls (New York, obtained from the *Drosophila* collection at Bowling Green, stock number 14012-0151.2), and *D. azteca* from Davis (California, collected by A. Latorre). Three belong to the *D. pseudoobscura* subgroup: *D. miranda* from Davis (California, collected by A. Latorre), *D. persimilis* from Cold Creek (California, obtained from the *Drosophila* collection at Bowling Green, stock number 14011-0111.0), and *D. pseudoobscura bogotana* from Bogotá (Colombia, obtained from the *Drosophila* collection at Bowling Green, stock number .14011-0121.35). The other eight species belong to the *D. obscura* subgroup: *D. ambigua* from Valencia (Spain, collected by A. Latorre), *D. bifasciata* from Akan-Ko (Japan, obtained from the *Drosophila* collection at Bowling Green, stock number 14012-0181.0), *D. guanche* from Tenerife (Canary Islands, Spain, collected by Dr. M. Monclús, University of Barcelona, Spain), *D. madeirensis* from Madeira Island (Portugal, collected by Dr. M. Monclús, University of Barcelona, Spain), *D. obscura* from Girona (Spain, collected by Dr. M. Monclús, University of Barcelona, Spain), *D. subobscura* from Zürich (Switzerland, collected by E. Hanschteck-Jüngen, Zoologisches Institut, Zürich), *D. subsilvestris* from unknown origin (supplied by Dr. M. Monclús, University of Barcelona, Spain), and *D. tristis* from Snery (Switzerland, collected by Dr. M. Monclús, University of Barcelona, Spain).

The mtDNA sequences of *D. yakuba* and *D. melanogaster,* used as references along this study, were obtained from Clary and Wolstenholme (1985) and Garesse (1988), respectively.

MtDNA Extraction, Cloning, and Sequencing. MtDNA was extracted by the methods of Latorre et al. (1986) and Afonso et al. (1988) with some modifications (Martínez et al. 1992). MtDNA samples were digested with *Eco*RI and the restriction fragments were separated on 0.8% low-temperature melting-point agarose gels with TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8) and ethidium bromide (0.5 μg/ml).

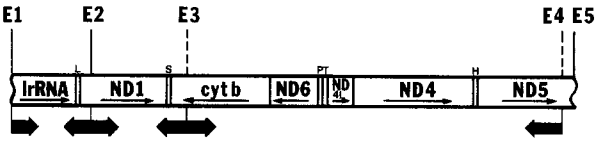

Fig. 1. Gene map of the mitochondrial DNA region partially sequenced. The areas sequenced in the 14 species of the *D. obscura* group are indicated by *thick arrows. Thin arrows* represent transcription direction of the genes. *Vertical lines* indicate the conserved (*continuous lines*) and variable (*dashed lines*) *Eco*RI sites used to clone this mitochondrial region.

Bands corresponding to the *Eco*RI fragments containing the region comprised between genes 16S-rRNA and NADH-dehydrogenase subunit 5 (positions 13,438 to 7,146 in the mtDNA sequence of *D. yakuba*) were excised from the gel, melted, and then cloned into plasmid vector pUC19 using *Escherichia coli* DH5α as a host (Sambrook et al. 1989).

Partial sequences of the genes 16S-rRNA, cytochrome b (cytb), NADH-dehydrogenase subunits 1 (ND1) and 5 (ND5), as well as complete sequences of the genes $tRNA^{Ser}$ and $tRNA^{Leu}$ were obtained by sequencing the ends of the cloned *Eco*RI fragments by Sanger's dideoxy sequencing method (Sanger et al. 1977) for denatured double-stranded plasmid DNA by using the T7 Sequencing kit (Pharmacia).

As not all the *Eco*RI sites are present in each species (see restriction maps in González et al. 1990; and Barrio et al. 1992), three synthetic oligonucleotides were used as internal primers in order to sequence the same homologous regions in all the species. Oligonucleotide (1) AATAAAGCATGAGTTAATAAATGAAATATAGC (positions 7,235 to 7,266) was used to sequence from the position homologous to the *Eco*RI site only present in the *D. pseudoobscura* subgroup species. Oligonucleotides (2) CTGGTCGAGCTCCAATTC (11,544 to 11,526) and (3) CGATCAATTCCTAATAAATTAGGAGGAGTAATTGC (11,361 to 11,395) were used to sequence in both directions from the homologous position to the *Eco*RI present in all species except in *D. azteca, D. ambigua, D. obscura, D. miranda,* and *D. subsilvestris,* and corresponding to position 11,462 in *D. yakuba.* For a detailed description of the different regions sequenced see Fig. 1.

The Genbank accession numbers are as follows: for *D. affinis,* U07272, U07273, U07274, and U07277; for *D. algonquin,* U07278, U07279, U07280, and U07281; for *D. azteca,* U07282, U07283, U07284, and U07285; for *D. subobscura,* U07286, U07287, U07288, and U07289; for *D. madeirensis,* U07290, U07291, U07292, and U07325; for *D. guanche,* U07293, U07294, U07295, and U07326; for *D. ambigua,* U07296, U07297, U07298, and U07299; for *D. obscura* U07300, U07301, U07302, and U07303; for *D. tristis,* U07304, U07305, U07306, and U07307; for *D. subsilvestris,* U07308, U07309, U07310, and U07311; for *D. bifasciata,* U07312, U07313, U07314, and U07315; for *D. miranda,* U07316, U07317, U07318, and U07319; for *D. pseudoobscura bogotana,* U07320, U07321, U07322, and U07323; and for *D. persimilis,* U07324, U07327, U07328, and U07329.

Phylogenetic Analysis. Sequences were aligned using the CLUSTAL V program (Higgins and Sharp 1988) and trees were constructed using the PHYLIP package v 3.41 and 3.5 (Felsenstein 1990), and the MEGA package v 1.0 (Kumar et al. 1993). *D. yakuba* and *D. melanogaster* sequences were used as outgroups in all the analyses. Tree-building procedures used were maximum parsimony (Fitch 1971), maximum-likelihood (Felsenstein 1981), and neighbor-joining (Saitou and Nei 1987). Genetic distances were estimated following Tamura's model (1992a).

Table 1. Percentages of nucleotide divergence (upper-right matrix) and standard errors (lower-left matrix) estimated according to Tamura (1992a) for the whole set of mitochondrial sequences from the 16 *Drosophila* analyzed

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *yakuba*	—	7.29	8.38	8.26	8.15	8.14	8.50	9.09	8.68	9.78	10.02	9.39	10.06	9.51	9.52	9.33
2. *melanogaster*	0.65	—	8.74	8.68	8.73	8.68	8.91	9.15	9.94	10.93	10.30	8.86	10.44	10.04	10.12	9.81
3. *affinis*	0.70	0.71	—	0.78	2.33	5.62	6.08	6.70	6.71	7.71	7.65	6.72	7.37	7.05	7.23	7.11
4. *algonquin*	0.69	0.71	0.20	—	2.06	5.68	6.03	6.30	6.77	7.83	7.77	6.55	7.25	6.75	7.11	6.88
5. *azteca*	0.68	0.71	0.36	0.33	—	5.51	6.15	6.14	6.88	7.90	8.12	6.06	7.71	6.87	7.40	7.17
6. *subobscura*	0.68	0.71	0.56	0.56	0.55	—	2.40	2.93	6.82	7.89	7.65	6.54	8.11	7.92	7.58	7.58
7. *madeirensis*	0.70	0.72	0.59	0.58	0.59	0.36	—	3.54	6.77	7.65	8.01	6.72	8.53	7.80	7.46	7.28
8. *guanche*	0.73	0.73	0.62	0.60	0.59	0.40	0.44	—	7.35	8.18	8.07	6.65	8.46	8.27	7.75	7.75
9. *ambigua*	0.71	0.77	0.62	0.62	0.63	0.62	0.62	0.65	—	2.50	4.92	5.81	8.08	8.11	7.71	7.59
10. *obscura*	0.76	0.81	0.67	0.68	0.68	0.68	0.67	0.69	0.37	—	5.91	6.28	8.92	8.96	8.25	8.25
11. *tristis*	0.77	0.78	0.67	0.67	0.69	0.67	0.68	0.69	0.53	0.58	—	7.09	8.79	8.72	8.37	8.07
12. *subsilvestris*	0.74	0.72	0.62	0.61	0.61	0.61	0.62	0.61	0.57	0.60	0.64	—	7.95	7.48	7.26	7.20
13. *bifasciata*	0.77	0.79	0.65	0.65	0.67	0.69	0.71	0.70	0.69	0.73	0.68	0.72	—	8.98	8.99	8.74
14. *miranda*	0.75	0.77	0.63	0.62	0.62	0.67	0.67	0.69	0.69	0.73	0.66	0.72	0.72	—	3.71	3.65
15. *ps. bogotana*	0.75	0.77	0.64	0.64	0.65	0.66	0.65	0.67	0.67	0.70	0.65	0.70	0.73	0.45	—	1.37
16. *persimilis*	0.74	0.76	0.64	0.62	0.64	0.66	0.64	0.67	0.66	0.70	0.65	0.69	0.71	0.45	0.27	—

Results

Sequence Analysis

Alignment of the partial mtDNA sequences by functional regions resulted in a total of 1,944 bp per species distributed as follows: 374 bp from 16S rDNA (homologous to the region between *D. yakuba* positions 13,432 and 13,139 and between 12,833 and 12,733); 65 bp from $tRNA^{Leu}$(CUN) gene (*D. yakuba* positions 12,755 to 12,691); 492 bp from NADH-dehydrogenase 1 gene (ND1, positions 12,680 to 12,225 and 11,777 to 11,706); 66 bp from $tRNA^{Ser}$(UCN) gene (positions 11,658 to 11,723); 501 bp from cytochrome b gene (cytb, positions 11,148 to 11,651); 408 bp from NADH-dehydrogenase 5 gene (ND5, positions 7,698 to 7,290); and the four short intergenic sequences located between these genes.

We observed nucleotide differences among these 14 *D. obscura* group species for 367 positions (18.9%), of which only 247 were phylogenetically informative (12.7%). Length variations are mainly confined to intergenic regions. Some insertions/deletions of single base pairs were also observed in the lrRNA gene regions corresponding to structural loops.

An important sequence difference is observed when comparing ND1 coding region of *D. yakuba* with those of the *D. obscura* group species. The 11 carboxy-terminal amino acids of the *D. yakuba* ND1 protein (Clary and Wolstenholme 1985) are absent in the species under study, due to the presence of a TAG or TAA stop codon. Thus, the overlap observed in *D. yakuba* between the 3′-ends of the genes $tRNA^{Ser}$(UCN) and ND1 encoded in opposite strands is lost in the *D. obscura* group species. This result is similar to that observed in *D. melanogaster* by Garesse (1988) and in species of the *D. nasuta* subgroup by Tamura (1992b), and is also supported by downstream deletions of adenines in the putative intergenic sequence (one in *D. melanogaster* and two in the *D. obscura* group species) which would bring the reading frame out of phase.

Sequence divergence for the whole region analyzed (Table 1) was determined according to Tamura (1992a), whose method accounts for the G+C and transversion-transition biases observed in *Drosophila* mtDNAs (Wolstenholme and Clary 1985; DeSalle et al. 1987; Tamura 1992b).

As expected, the most-conserved region corresponds to the lrRNA gene, where only 28 positions were variable (7.5%). For the protein genes the percentage of polymorphic sites is 19.1% for ND1, 23.5% for ND5, and 24.8% for cytb, approximately of the same order. However, the percentage of variable positions at the amino acid level is clearly lower for ND1 (5.5%) than for ND5 (8.1%) and cytb (9.6%).

A strong transition bias for nucleotide substitutions between closely related species is shown in Table 2. As could be seen, the ratio between transitions and transversions falls from 14–12:1 for closely related species (*affinis-algonquin* and *ps. bogotana-persimilis*) to <1:1 for the most divergent sequences. The loss of the transition bias has been explained by the constant accumulation of transversions that, although occurring relatively rarely, tend to erase the record of transitions (Brown et al. 1982; DeSalle et al. 1987). As is shown in Fig. 2, the divergence due to transversion (Table 2) is linearly proportional to the total divergence (Table 1).

Base Composition

Table 3 gives the total G+C content for the 1,944 bp sequenced in all 14 species, as well as the nucleotide frequencies at third-codon positions for the partial sequences of the three protein-coding genes. Although the G+C content is very similar in all the species (ranging

Table 2. Nucleotide divergence (%) due only to transversions (above diagonal) determined according to Tamura (1992a), and total number of transversions/transitions (below diagonal)

Species	1	2	3	4	5	6	7	8
1. *yakuba*	—	3.88	5.07	5.12	5.01	5.18	5.24	5.64
2. *melanogaster*	72/61	—	5.30	5.35	5.47	5.41	5.47	5.76
3. *affinis*	95/59	97/61	—	0.05	0.68	3.32	3.26	3.88
4. *algonquin*	96/56	98/59	1/14	—	0.63	3.26	3.21	3.82
5. *azteca*	93/56	100/58	13/31	12/27	—	3.15	2.99	3.49
6. *subobscura*	96/53	99/58	63/42	62/44	60/43	—	0.36	0.94
7. *madeirensis*	97/58	100/61	62/51	61/51	57/57	7/38	—	0.99
8. *guanche*	103/62	105/61	72/51	71/45	65/48	19/38	20/48	—
9. *ambigua*	92/67	104/74	66/58	67/58	69/58	69/56	66/58	74/62
10. *obscura*	92/84	105/89	68/72	67/75	67/76	71/73	70/70	72/77
11. *subsilvestris*	103/67	96/64	61/62	62/58	66/55	64/57	63/61	69/54
12. *tristis*	95/86	107/77	68/71	69/72	73/74	71/69	72/74	72/75
13. *bifasciata*	104/77	103/83	67/70	68/67	72/69	81/67	80/75	84/70
14. *miranda*	108/64	115/65	81/51	82/45	85/43	89/56	90/53	92/59
15. *ps. bogotana*	99/73	106/75	76/59	77/56	76/61	78/61	77/60	79/63
16. *persimilis*	101/68	106/70	76/57	77/52	76/57	78/61	77/57	79/63

Species	9	10	11	12	13	14	15	16
1. *yakuba*	4.90	4.95	5.59	5.07	5.64	5.88	5.35	5.47
2. *melanogaster*	5.70	5.76	5.24	5.88	5.64	6.34	5.82	5.82
3. *affinis*	3.49	3.65	3.26	3.65	3.43	4.21	3.93	3.93
4. *algonquin*	3.54	3.60	3.32	3.71	3.49	4.27	3.99	3.99
5. *azteca*	3.65	3.60	3.54	3.93	3.82	4.50	3.99	3.99
6. *subobscura*	3.71	3.77	3.38	3.77	4.33	4.78	4.16	4.16
7. *madeirensis*	3.54	3.71	3.32	3.82	4.27	4.84	4.10	4.10
8. *guanche*	3.93	3.88	3.71	3.88	4.55	5.01	4.27	4.27
9. *ambigua*	—	0.47	2.55	1.74	3.49	4.27	3.77	3.77
10. *obscura*	10/38	—	2.50	1.79	3.65	4.21	3.71	3.71
11. *subsilvestris*	49/59	47/68	—	2.93	3.82	3.60	3.21	3.10
12. *tristis*	34/58	34/74	55/74	—	3.88	4.10	3.71	3.82
13. *bifasciata*	67/81	69/92	72/73	75/86	—	5.12	4.84	4.95
14. *miranda*	81/68	80/83	69/69	79/81	96/68	—	0.99	0.99
15. *ps. bogotana*	72/70	71/80	62/72	72/82	91/73	19/50	—	0.07
16. *persimilis*	72/68	71/80	60/73	74/75	93/67	19/49	2/24	—

from 23.2% in *D. subobscura* to 25.7% in *D. obscura*), a clear, strand-specific change of nucleotide frequencies at third-codon positions is observed for some of the species. Thus, a higher frequency of G in the ND1 and ND5 genes, and of C in the cytb gene (encoded on the opposite strand), is apparent in some species of the *obscura* subgroup (*D. ambigua, D. obscura, D. tristis, D. subsilvestris,* and *D. bifasciata*).

Phylogenetic Analysis

In order to perform the different phylogenetic analyses, all the available sequence data were combined into a single set (1,944 bp per species). This procedure is correct for maximum-parsimony and maximum-likelihood methods according to the assumption of independence among characters of these methods (Swofford and Olsen 1990), and has been proved to increase phylogenetic signal (Cracraft and Helm-Bychowski 1991). In the case of the distance matrix approach, each region should be treated separately due to the existence of different rates of nucleotide substitution (data not shown) because the internodal distances are dependent on the rate of evolution (Li and Gouv 1990). Nevertheless, as can be seen in Fig. 3, the nonrandom distribution of substitutions in mitochondrial genes (Thomas and Beckenbach 1989) is a problem in estimating evolutionary relationships from short partial sequences. As Martin et al. (1990) pointed out, this problem could be solved by including in the analysis larger sections of DNA. Because the phylogenies obtained from the different mitochondrial regions (Fig. 3) showed incongruent relationships for some of the species (e.g., *D. miranda* with the RNA data or *D. azteca* with the ND1 data), we decided also to combine all the available data to perform the distance-based methods.

Phylogenetic trees based on pairwise distances (Fig. 3–5), parsimony (Fig. 6), as well as maximum-likelihood methods (Fig. 7) resulted in a similar interpretation of the

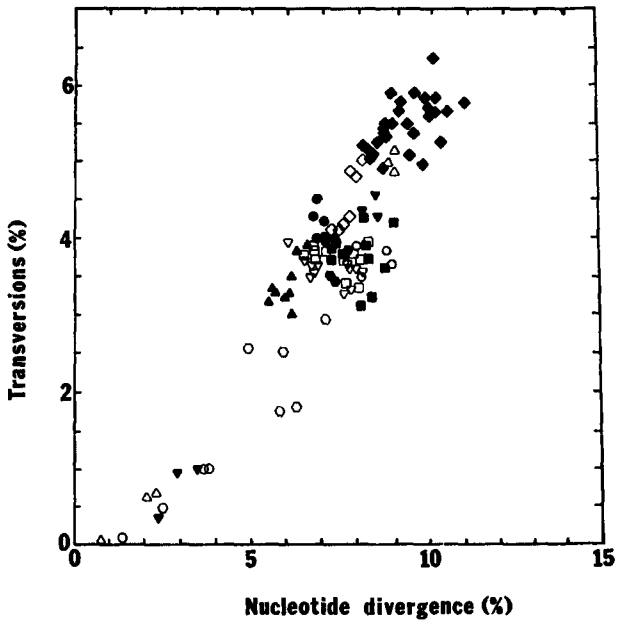

Fig. 2. Divergence due to transversions plotted against nucleotide divergence (both divergences corrected for multiple substitutions according to Tamura 1992a) for all comparisons between species of the different subgroups and lineages of the *D. obscura* group (*different symbols*), and between these species and the two representatives of the *D. melanogaster* subgroup (♦). Symbols for the different comparisons within *D. obscura* group are as follows: lower left △, within *affinis* subgroup (*aff*); ⬭, within *obscura* complex (*obs*), including *D. obscura, D. ambigua, D. tristis,* and *D. subsilvestris*. Lower left ○, with *pseudoobscura* subgroup (*pse*). Lower left ▼, within *subobscura* complex (*sub*), formed by *D. subobscura, D. guanche,* and *D. madeirensis*. ◇, between *pse* and *sub*. ⬬, between *aff* and *D. bifasciata* (*bif*). ▽, between *aff* and *obs*. ●, between *aff* and *pse*. ▲, between *aff* and *sub*. Upper right ○, between *bif* and *obs*. Upper right △, between *bif* and *pse*. Upper right ▼, between *bif* and *sub*. ■, between *obs* and *pse*. □, between *obs* and *sub*.

phylogenetic relationships between the *D. obscura* species. The *D. obscura* species studied are grouped in, at least four well-defined clusters, the already-accepted *pseudoobscura* and *affinis* subgroups and two monophyletic complexes (*ambigua-obscura-tristis-subsilvestris*—namely, the *obscura* complex; and *subobscura-madeirensis-guanche*—namely, the *subobscura* complex) belong to the heterogeneous *obscura* subgroup. The branching position of *D. bifasciata* is not well established according to the confidence limits of the phylogenetic reconstructions (less than 70% of the bootstrap according to Hillis and Bull 1993). Nevertheless, this species always clustered with the *obscura* complex, except when only transversions were considered (Fig. 5). In this phylogenetic reconstruction, *D. bifasciata* and also *D. subsilvestris* were shown as independent lineages.

Although the whole *obscura* group is significantly monophyletic, only the relationships between closely related species were well established, and the relationships between subgroups, complexes, and lineages remain unclear. Different topologies relating the *pseudoobscura* and *affinis* subgroups, *subobscura* and *obscura* complexes, and the *D. bifasciata* lineage were obtained by applying the different tree-making methods. When the 15 different trees representing all the possible branching orders among the subgroups and complexes (*D. bifasciata* was included in the *obscura* complex) were compared by the test on maximum parsimony of Templeton (1983; modified by Felsenstein 1985a,b) and by the test on maximum likelihood of Kishino and Hasegawa (1989), the differences were not significant (Table 4). Of the 15 trees, only four were considered worse than the most parsimonious tree according to the Templeton's test, but none according to the Kishino and Hasegawa's.

Discussion

The *D. obscura* group is one of the best-studied *Drosophila* groups with respect to phylogenetic relationships. Nonetheless, two important questions remain controversial—the phylogenetic relationships between lineages and the monophyletic or polyphyletic character of the *D. obscura* subgroup.

As has been seen in the present study, the *D. obscura* group appears as a group formed by four or five lineages, two of them giving rise to the monophyletic *pseudoobscura* and *affinis* subgroups, and the other to the clearly polyphyletic *obscura* subgroup (i.e., the *subobscura* and *obscura* complexes and the *D. bifasciata* lineage). Nevertheless, when we try to determine the relationships among the different lineages, any possible relationship could be valid. It could be thought that the impossibility to decipher accurately phylogenetic relationships between subgroups, complexes, or lineages within the *D. obscura* group could be simply the result of an insufficient number of nucleotides examined. Furthermore, it seems more likely that the lack of resolution is actually evidence for a rapid phyletic radiation within the *D. obscura* group. Short internode lengths and similar levels of sequence divergence among taxa have been suggested as consistent with hypotheses of rapid origin and radiation of lineages (Kraus and Miyamoto 1991). Previous phylogenetic studies based on different approaches (see Introduction) to the resolution of the relationships among members of the *D. obscura* group seem to be contradictory. However, when they are analyzed in detail, we can observe that the rapid phyletic radiation hypothesis is not incongruent with them. In the case of phylogenetic analyses based on allozyme polymorphism some studies support the monophyletic character of the *obscura* subgroup (Lakovaara et al. 1972, 1976; Lakovaara and Keränen 1980; Pinsker and Buruga 1982; Loukas et al. 1984), and others showed such a subgroup as polyphyletic (Marinkovic et al. 1978; Carbrera et al. 1983; Cariou et al. 1988). In all of them, the genetic distances within the

Table 3. G+C content for the sequences obtained from each species and nucleotide composition (%) in the third positions of the codons sequenced in the three protein-coding genes[a]

	Total	ND1 (163)				cytb (166)				ND5 (136)			
Species	%G+C	A3	T3	C3	G3	A3	T3	C3	G3	A3	T3	C3	G3
yakuba	23.9	44.8	51.5	1.2	2.5	46.4	45.8	6.6	1.2	42.6	49.3	2.2	5.9
melanogaster	23.6	38.7	54.0	0.0	7.4	47.6	46.4	4.8	1.2	48.5	47.8	0.7	2.9
affinis	23.9	42.3	54.6	0.6	2.5	45.8	48.2	5.4	0.6	42.6	51.5	0.7	5.1
algonquin	23.6	41.1	55.8	0.0	3.1	45.8	48.8	4.8	0.6	43.4	52.2	0.0	4.4
azteca	23.4	42.3	54.6	0.6	2.5	45.2	50.0	4.2	0.6	48.5	48.5	0.0	2.9
subobscura	23.2	41.7	54.6	0.6	3.1	47.0	48.8	2.4	1.8	46.3	46.3	2.2	5.1
madeirensis	23.6	38.7	52.8	1.8	6.7	48.8	47.0	4.2	0.0	49.3	45.6	0.7	4.4
guanche	23.3	41.7	51.5	1.8	4.9	48.2	46.4	4.8	0.6	50.0	46.3	0.7	2.9
ambigua	24.8	41.1	52.1	0.6	6.1	47.0	40.4	11.4	1.2	43.4	46.3	0.0	10.3
obscura	25.7	39.3	52.1	1.2	7.4	45.8	40.4	12.0	1.8	41.2	45.6	2.2	11.0
tristis	24.2	39.3	52.1	0.6	8.0	41.6	47.0	9.0	2.4	47.8	47.8	0.7	3.7
subsilvestris	25.0	41.1	52.1	0.6	6.1	47.6	41.0	10.8	0.6	39.0	50.7	1.5	8.8
bifasciata	24.6	39.9	49.7	1.2	9.2	48.8	44.6	5.4	1.2	42.6	47.1	0.0	10.3
miranda	23.6	46.6	49.1	1.8	2.5	45.2	48.8	5.4	0.6	42.6	50.7	0.7	5.9
ps. bogotana	24.3	42.9	49.1	1.8	6.1	44.6	45.2	9.0	1.2	42.6	50.0	1.5	5.9
persimilis	24.1	42.3	49.7	1.2	6.7	44.6	46.4	9.0	0.0	43.4	51.5	0.0	5.1

[a] The total number of codons analyzed for each region is shown in parentheses

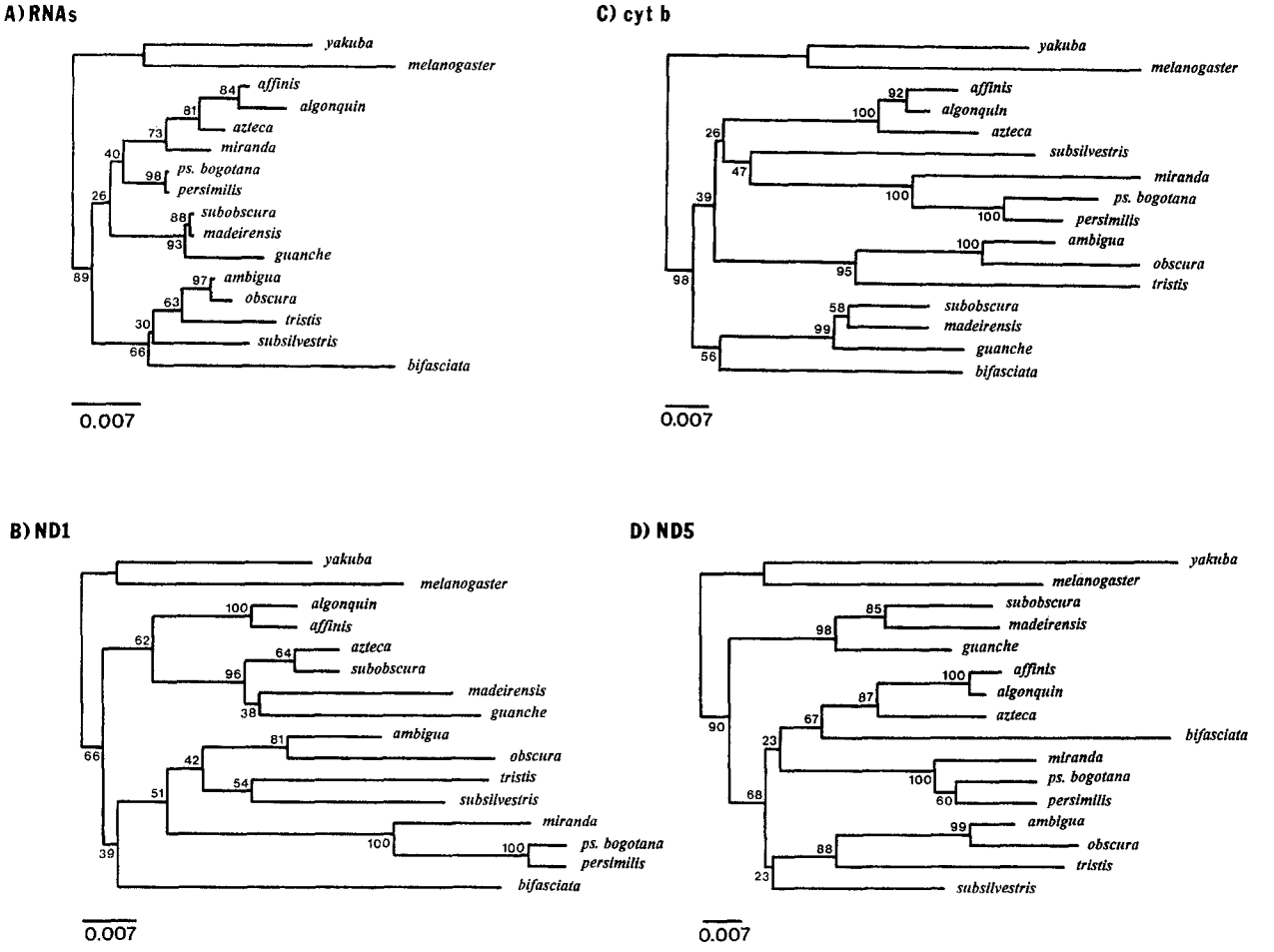

Fig. 3. Neighbor-joining trees of 14 species of the *Drosophila obscura* group based on Tamura's distance from the composite sequences of the three mitochondrial RNA regions sequenced (panel **A**) and the partial sequences of each of the three mitochondrial proteins genes analyzed (panels **B–D**). *D. yakuba* and *D. melanogaster* are included as outgroups. Branch lengths are proportional to the scale given in substitutions per nucleotide. The bootstrap values on the nodes are percentages for 500 replicates.

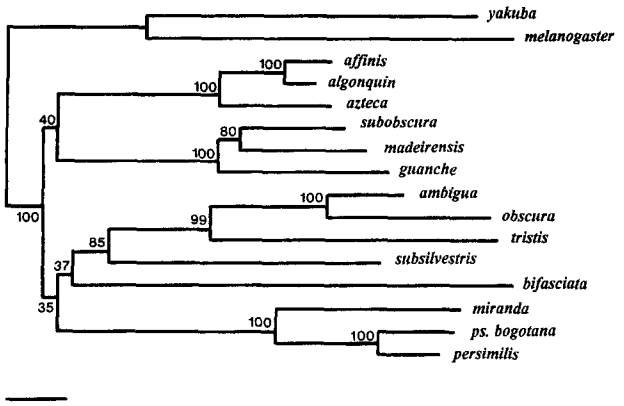

Fig. 4. Neighbor-joining tree based on total nucleotide divergence corrected according to Tamura's method, determined by using the combination of all available sequences of the different mitochondrial regions analyzed in 14 species of the *D. obscura* group. *D. yakuba* and *D. melanogaster* are included as outgroups. Branch lengths are proportional to the scale given in substitutions per nucleotide. The bootstrap values on the nodes are percentages for 1,000 replicates.

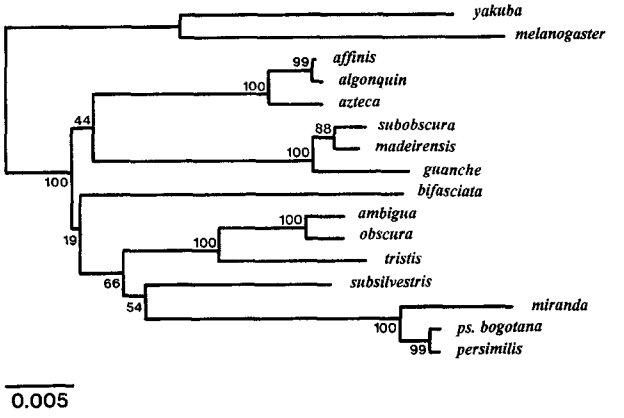

Fig. 5. Neighbor-joining tree based on nucleotide divergences due only to transversion substitutions corrected according to Tamura's method from the combination of all available sequences from 14 species of the *D. obscura* group. *D. yakuba* and *D. melanogaster* are included as outgroups. Branch lengths are proportional to the scale given in transversions per nucleotide. The bootstrap values on the nodes are percentages for 1,000 replicates.

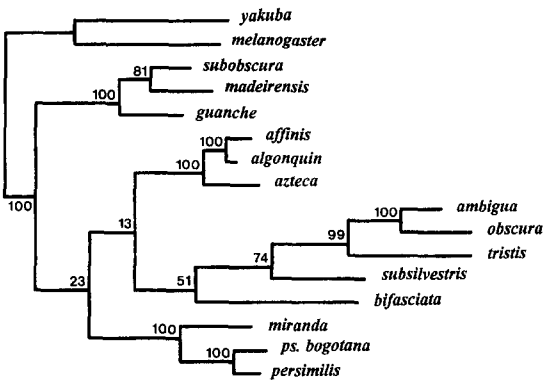

Fig. 6. Most parsimonious tree that required a total of 885 substitutions (deletions were not included in the analysis). The tree was obtained for the whole set of mitochondrial sequences of 14 *D. obscura* group species using the maximum-parsimony algorithm with bootstrap resampling (DNAPARS and SEQBOOT programs of the PHYLIP package, Felsenstein 1990). The tree was rooted by using *D. yakuba* and *D. melanogaster* as outgroups. Branch lengths are proportional to the scale given in number of substitutions. The bootstrap values on the nodes are percentages for 1,000 replicates.

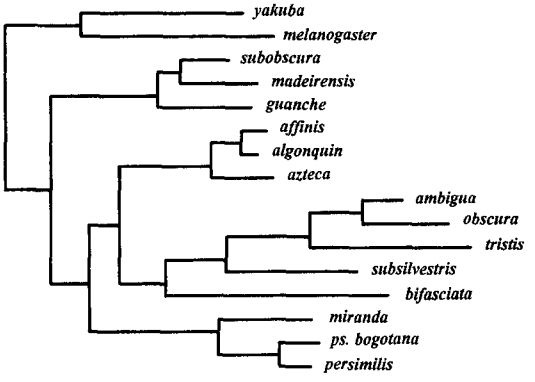

Fig. 7. Maximum-likelihood tree obtained for the whole set of mitochondrial sequences of 14 *D. obscura* group species using the DNAML algorithm (PHYLIP package). The tree was rooted by using *D. yakuba* and *D. melanogaster* as outgroups. Branch lengths are proportional to the scale given in number of substitutions per nucleotide. Identical substitution rates were assumed for all nucleotide positions. Different transition/transversion rates were assayed and a rate of 1.0 gave the maximum likelihood.

obscura subgroup are not much lower than those between subgroups, and the associated standard errors allow other possible interpretations of the phylogenetic relationships, including the rapid phyletic radiation scenario proposed in this study.

Other phylogenetic analyses based on molecular approaches are also compatible with the rapid radiation hypothesis. Analyses based on mitochondrial restriction analysis (Latorre et al. 1988; González et al. 1990; Barrio et al. 1992), on DNA-DNA hybridization (Goddard et al. 1990), or on nuclear (Marfany and Gonzàlez-Duarte 1993) or mitochondrial (Ruttkay et al. 1992; Beckenbach et al. 1993) sequences have similar difficulties in the assessments of the phylogenetic relationships between subgroups, clusters, or lineages of the *D. obscura* group.

The rapid phyletic radiation hypothesis could explain the discrepancy of the phylogenies obtained on the basis of different approaches with respect to the relationships between the *obscura* group lineages. According to Pamilo and Nei (1988), the probability of obtaining a gene tree different from the species tree is quite high when the interval time between species splitting is short. If the speciation events that gave rise to the different lineages of the *obscura* group occurred in short intervals of time,

Table 4. Templeton's test of maximum parsimony and Kishino and Hasegawa's (1989) test of maximum likelihood applied to the 15 different trees that represent all the possible branching orders among subgroups and complexes of the *D. obscura* group (O, *obscura;* A, *affinis;* P, *pseudoobscura;* S, *subobscura;* M, *melanogaster* group)[a]

		Templeton's test of maximum parsimony				Kishino and Hasegawa's test of maximum likelihood			
Tree	Topology	Steps	Diff. steps	S.D.	Worse?	Ln L	Diff. Ln L	S.D.	Worse?
1	((((O,A),P),S),M)	885	—	—	—	−7,513.15	—	—	—
2	(((O,P),(S,A)),M)	887	2	5.10	No	−7,516.85	−3.70	16.67	No
3	((((O,P),A),S),M)	887	2	3.47	No	−7,515.62	−2.47	8.72	No
4	((((O,P),S),A),M)	890	5	4.80	No	−7,526.54	−13.39	14.43	No
5	((((S,A),O),P),M)	889	4	5.10	No	−7,516.90	−3.76	16.54	No
6	((((S,A),P),O),M)	889	4	5.29	No	−7,517.52	−4.38	16.89	No
7	(((S,P),(O,A)),M)	891	6	2.83	Yes	−7,526.98	−13.83	7.22	No
8	((((S,P),A),O),M)	893	8	4.24	No	−7,534.13	−20.99	11.70	No
9	((((S,P),O),A),M)	894	9	4.12	Yes	−7,532.31	−19.16	12.35	No
10	((((O,A),S),P),M)	891	6	2.83	Yes	−7,525.82	−12.67	7.63	No
11	(((O,S),(A,P)),M)	891	6	4.24	No	−7,528.73	−15.59	13.51	No
12	((((O,S),A),P),M)	894	9	4.58	Yes	−7,529.48	−16.33	14.11	No
13	((((O,S),P),A),M)	892	7	5.00	No	−7,525.62	−12.47	15.31	No
14	((((A,P),O),S),M)	890	5	3.00	No	−7,522.58	−9.43	6.59	No
15	((((A,P),S),O),M)	891	6	4.24	No	−7,532.84	−19.69	12.24	No

[a] The differences obtained when compared with the lowest values in both tests are Diff. steps and Diff. Ln L, respectively. S.D. is the standard deviation estimated and used to test if such differences are statistically significant. For more details see results

the ancestral polymorphism could be randomly maintained or lost in the different lineages. As a consequence of that, the phylogenetic reconstructions based on different genes, regions, or genomes show different relationships between lineages, corresponding to the different gene trees and not to the species tree.

In addition, the rapid radiation hypothesis is also coherent with the evolutionary scenario proposed by Throckmorton (1975) for the origin of the *D. obscura* group. On the basis of biogeographical, morphological, and other considerations, Throckmorton argued that the divergence of the *obscura* group occurred in the Palearctic region during the Miocene (20–25 Mya), simultaneously with the rapid expansion of the deciduous forest through the temperate zone.

Nevertheless, another possible explanation to the impossibility of solving the elusive phylogenetic relationships between subgroups and lineages within the *D. obscura* group would come from the effect that the special evolutionary dynamics of the mitochondrial genome in *Drosophila* could have on the phylogenetic reconstructions. We tried to overcome the fast saturation effect due to both A+T and transition-transversion biases and a high substitution rate, observed by DeSalle et al. (1987), and confirmed by other authors (Barrio et al. 1992; Liu and Beckenbach 1992; Tamura 1992b; Beckenbach et al. 1993; and the present study) by using Tamura's method of sequence divergence estimation to correct the effect of such biases. However, reconstructions based on Tamura's distances gave the same elusive phylogenetic relationships as reconstructions obtained according to other tree-making methods that do not consider this kind of corrections.

Another important effect on the estimation of the phylogenetic relationships between the *D. obscura* group species could come from the different compositional constraints observed in the mitochondrial genes coding for proteins. Saccone et al. (1993) demonstrated that when homologous sequences significantly differ in base composition, an erratic branching order and/or wrong evaluation of the evolutionary rates could be obtained. As was indicated, some species of the *obscura* group showed significantly different strand-specific nucleotide composition in the third codon position. By analyzing the COII (gene encoded in the same strand as cytb, and opposite to ND1 and ND5) sequence data for the *D. obscura* group species from Beckenbach et al. (1993), the same strand-specific composition bias in the third codon position is observed in *D. ambigua* (9.2% of C vs 4.4% ± 1.4% in the other species), the only species from the *obscura* complex analyzed by those authors. The same bias has been reported in the *D. nasuta* species subgroup by Tamura (1992b), who suggested that the pattern of nucleotide substitution for *Drosophila* mtDNA may vary from species to species. Tamura also suggested that this strand-specific bias could be related to the replication mechanism in the *Drosophila* mtDNA. Additional studies should be carried out in order to understand the complexity of the evolutionary dynamics of the *Drosophila* mtDNA.

In relation to the controversy about the monophyletic or polyphyletic character of the *D. obscura* subgroup

(see Introduction) our data show a clearly polyphyletic origin. Sequence data from either mitochondrial (Ruttkay et al. 1992; Beckenbach et al. 1993) or nuclear genes (Marfany and Gonzàlez-Duarte 1993) are also congruent with the phylogenies obtained in the present study. In those cited studies, the species of the so-called *D. obscura* complex (*D. obscura* and *D. tristis* in the first study and *D. ambigua* in the other two) are placed with the species of the *pseudoobscura,* and never as the sister taxon of the so-called *subobscura* complex (*D. subobscura, D. madeirensis,* and *D. guanche*). However, as occurs in the present work, these relationships were not statistically supported by bootstrapping. The complexity of the *obscura* subgroup was also apparent in the chromosomal analysis of these species and two representatives of the African *microlabis* subgroup carried out by Brehm et al. (1991) and Brehm and Krimbas (1990, 1992, 1993). They proposed the existence of two well-differentiated clusters within the *obscura* subgroup, *obscura* and *subobscura* (corresponding to our complexes), and *D. subsilvestris,* which, although related to the *obscura* cluster, stands apart.

On the basis of the evidence reported in the present study, different lineages of the *obscura* group were as a consequence of the adaptation and colonization of the new habitats generated by the expansion of the temperate deciduous forest throughout the Palearctic region. The present *obscura* subgroup is a heterogeneous cluster of species descendant from different lineages originated during this rapid radiation, and the Nearctic subgroups (*pseudoobscura* and *affinis*) are monophyletic groups of species originated later from ancestors from one (according to Goddard et al. 1990; and Beckenbach et al. 1993) or two (according to the present study) lineages that colonized the Nearctic subregion during the posterior expansion of the deciduous forest to North America through the Bering strait. The same conclusion could be reached for the Ethiopian species of the *microlabis* subgroup. Results from previous analyses of two of these species (Cariou et al. 1988; Ruttkay et al. 1992; Brehm and Krimbas 1993) indicate that these species form part of a monophyletic lineage which colonized the Ethiopian region in the past (Cariou et al. 1988).

Accordingly, the subdivision of the *D. obscura* group could be maintained by considering the existence of the paraphyletic *obscura* subgroup and the monophyletic *affinis, microlabis,* and *pseudoobscura* subgroups, or revised by raising the different clusters, complexes, or lineages of the *obscura* subgroup to the rank of subgroups. This last proposal is problematic due to the difficulty in defining the new subgroups, as a consequence of the ambiguous position of some of the species. The only clear new taxon could be the monophyletic *subobscura* subgroup formed by *D. subobscura, D. guanche,* and *D. madeirensis.* The definition of the new *subobscura* subgroup is also supported by morphological as well as chromosomal characteristics. Buzzatti-Traverso and Scossiroli (1955) studied morphological similarities between species of the *D. obscura* group and concluded that *D. subobscura* (the other two species were unknown in 1955) differs more from each of the Palearctic species of the *obscura* subgroup than these differ from each other. Lakovaara and Këranen (1980) published phylogenetic trees based on Buzzatti-Traverso and Scossiroli's morphological data and reached similar conclusions. Finally, Lakovaara and Saura, in their revision of the *D. obscura* subgroup (1982), pointed out the difficulties in assigning *D. subobscura* and *D. guanche* (*D. madeirensis* was undescribed by then) to any cluster according to morphological traits. Since the description of *D. guanche* and *D. madeirensis,* many authors have mentioned the close relationships between these species and *D. subobscura,* and their inclusion in a separate cluster within the *obscura* subgroup on the grounds of morphological, chromosomal, allozyme, and molecular data (Lakovaara and Këranen 1980; Cabrera et al. 1983; Loukas et al. 1984; Cariou et al. 1988; González et al. 1990; Barrio et al. 1992; Brehm and Krimbas 1992, 1993). However, the most important character that differentiates these three species from the other *D. obscura* species is the haploid chromosome complement formed by five rodlike chromosomes, one of which corresponds to the X chromosome, and one dot (this could be absent in *D. madeirensis*) (Krimbas and Loukas 1984). In the *D. obscura* group, the presence of an achrocentric X chromosome in common with that of *D. melanogaster* has been reported only in these three species, which led Lakovaara and Saura (1982) to postulate the primitive condition of this character.

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