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On the Evolution of Dopa decarboxylase (Ddc) and Drosophila Systematics

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Abstract. We have sequenced most of the coding region of the gene Dopa decarboxylase (Ddc) in 24 fruitfly species. The Ddc gene is quite informative about Drosophila phylogeny. Several outstanding issues in Drosophila phylogeny are resolved by analysis of the Ddc sequences alone or in combination with three other genes, Sod, Adh, and Gpdh. The three species groups, melanogaster, obscura, and willistoni, are each monophyletic and all three combined form a monophyletic group, which corresponds to the subgenus Sophophora. The Sophophora subgenus is the sister group to all other Drosophila subgenera (including some named genera, previously considered outside the Drosophila genus, namely, Scaptomyza and Zaprionus, which are therefore downgraded to the category of subgenus). The Hawaiian Drosophila and Scaptomyza are a monophyletic group, which is the sister clade to the virilis and repleta groups of the subgenus Drosophila. The subgenus Drosophila appears to be paraphyletic, although this is not definitely resolved. The two genera Scaptodrosophila and Chymomyza are older than the genus Drosophila. The data favor the hypothesis that Chymomyza is older than Scaptodrosophila, although this issue is not definitely resolved. Molecular evolution is erratic. The rates of nucleotide substitution in 3rd codon position relative to positions 1 + 2 vary from one species lineage to another and from gene to gene.

Key words: Dopa decarboxylase (Ddc) — Divergence — Drosophila — Scaptodrosophila — Chymomyza — Zaprionus — Scaptomyza

Introduction

The received classification of the Drosophilidae (e.g., Wheeler 1981) is inconsistent with the phylogenetic relationships among the species, whether these are based on morphology (Throckmorton 1975; Grimaldi 1990) or molecular data (Kwiatowski et al. 1997; Powell 1997).

Throckmorton (1975) advanced a comprehensive scheme of the phylogenetic relationships in the Drosophilidae and showed that paraphyly is widespread among the various groups. However, he did not make any attempt to bring the classification of the Drosophilidae in correspondence with his hypothesis of phylogenetic relationships. Grimaldi (1990) has more recently constructed a phylogeny of the family, using a number of morphological characters and relying on strict cladistic methods, concluding also that Wheeler’s classification implies extensive paraphyly. Grimaldi (1990) has accordingly proposed a new classification of the Drosophilidae, which is consistent with his hypothesis of phylogenetic relationships. Grimaldi’s phylogenetic hypothesis displays important disparities with Throckmorton’s and has been shown also to be inconsistent with extensive molecular data (DeSalle 1992; Thomas and Hunt 1993; Kwiatowski et al. 1994, 1997; Powell 1997; Remsen and DeSalle 1998).

One particularly noteworthy discrepancy between Grimaldi’s and Throckmorton’s phylogenies concerns the position of the subgenus Sophophora (which includes
D. melanogaster). Grimaldi (1990) considers Sophophora to be a sister-taxon of the subgenus (s.g.) Drosophila, which together with the s.g. Dorsilopa, would make up the genus Drosophila. In contrast, Throckmorton (1975) considered the s.g. Drosophila to be phylogenetically closer to several genera and subgenera (such as Zapronius, Samaoa, Dorislopha, Hirtodrosophila, and Scaptomyza) than to Sophophora. Molecular data have, on the whole, favored Throckmorton’s rather than Grimaldi’s hypothesis in this respect (e.g., Kwiatowski et al. 1994, 1997; Russo et al. 1995; Powell 1997; Remsen and DeSalle 1998) but have left unresolved the relationships among several genera and subgenera (and brought together in question whether the willistoni group, usually included in the subgenus Sophophora, may actually be a sister taxon to the Drosophila genus (e.g., Pelandakis and Solignac 1993). These unsettled issues are significant, particularly the phylogenetic position of Sophophora, because this subgenus includes D. melanogaster, which is so extensively used as a model species for many evolutionary, developmental, and molecular biology investigations.

In this paper we study the phylogenetic relationships among 24 species of the family Drosophilidae, using the nucleotide sequences of Dopa decarboxylase (Ddc), a nuclear gene involved in morphological differentiation and in the production of the neurotransmitters, dopamine and serotonin. The product of this gene, DDC, catalyzes the decarboxylation of dopa to dopamine and is essential for the sclerotization and melanization of the cuticle (Wright 1996, and references therein). This gene is conserved between Drosophila and humans and is expressed in the central nervous system (CNS) as well as in the peripheral nervous system of insects and mammals (Wright et al. 1982; Konrad et al. 1993; Wang and Marsh 1995; Wang et al. 1996; Wright 1996). The only Drosophilid nucleotide sequence of Ddc already published is that of D. melanogaster (Eveleth et al. 1986). We have sequenced this gene in another 22 Drosophilid species and in the medfly Ceratitis capitata. The Ddc gene has been found to be a highly appropriate marker for phylogenetic analysis in a subfamily of Lepidoptera that arose within the last 20 million years (Fang et al. 1997). Comparison of D. melanogaster Ddc with that of other animals (such as mosquito, moth, and some mammals) indicates that it can be informative at deeper taxonomic levels as well.

Materials and Methods

Species. The 24 species studied are listed in Table 1. The Drosophilidae species originate from the National Drosophila Species Resource Center (Yoon 1996); for the source of Ceratitis capitata see Kwiatowski et al. (1992). We list as Drosophila subgenera some taxa classified as genera by Wheeler (1981), but Scaptodrosophila as a genus, following Grimaldi (1990) and Kwiatowski et al. (1994, 1997).

DNA Preparation and Sequencing. Genomic DNA was extracted following the procedure described by Palumbi et al. (1991). The published sequences from a moth (Manduca sexta; GenBank U03909), a fly (Drosophila melanogaster; X04661), and a mosquito (Aedes aegypti; U27581) were used to design PCR primers. Two slightly different methods (a and b) were followed for amplification and sequencing. Method a was used for species 1—12, 14, 22, and 24; method b, for species 13 and 15—23 (see Table 1 for ID numbers; Scaptodrosophila was analyzed with both methods).

Method a. The amplifying primers were 5′-ACACTGTAGCCGCCTACCTGCCACC-3′ (APF; forward primer), and 5′-CGGCTGTGTTGACCTCTTTAAGCGAAGACCAC-3′ (APR; reverse primer). The amplified fragment is 963—966 bp long and encompasses most of the Ddc exon 4 gene, accounting for 68% of the total Ddc coding sequence in D. melanogaster. The shorter fragments (963 rather than 966 bp) are due to a 3-bp deletion shared by all species of the Sophophora subgenus. All PCR reactions were conducted as described by Kwiatowski et al. (1991). Amplification parameters were as follows: initial denaturation at 94°C for 5 min, followed by 31 cycles of denaturation, annealing, and extension at 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min, respectively; after 30 cycles, the reaction was additionally kept at 72°C for 7 min to complete extension. PCR products were purified with Wizard PCR prep DNA purification system (Promega corporation) and cloned using the TA cloning kit (Invitrogen, San Diego, CA). DNA sequencing was done by the dideoxy-chain-termination technique with Sequenase Version 2.0 T7 DNA polymerase (Amersham Life Sciences Inc., USA) using 32P-labeled dATP. The internal sequencing primers were as follows: A1F, 5′-ATAGGCAACGTGTTGGCTA-3′; A2F, 5′-ATCCAGATGGGAYGARCCAC-3′; A3F, 5′-TGGAGATTCTGAGCTGTCGGCACTGTTG-3′; A1R, 5′-AGAGCCACCACAGTGGAATACTACGTGGC-3′; A2R, 5′-TCAAGGTSSACCCAGATCCA-3′; and A3R, 5′-ASCCACATGCGCAGACGTC-3′.

Method b. The amplifying primers were 5′-GAYATYAGARCGNNTSATCATGCKCGK-3′ (BPF; forward primer) and 5′-TSRGTTAGACNGRCADAYKGGCAGCAT-3′ (BPR; reverse primer). The amplified fragments were longer than with method a, but we analyze here only the nucleotide sequence corresponding to the PCR fragments of method a. PCR amplifications were performed in a 100-μl volume of the ExTAKARA buffer, containing 2.5 U of ExTAKARA Taq polymerase, 0.2 mM dNTP (all from TAKARA), a 0.5 μM concentration of primers, and 3 μl of template DNA. The cycling parameters for the amplification were as follows: initial denaturation at 95°C for 5 min, followed by 31 cycles with denaturation for 30 s at 95°C, annealing for 30 s at 59°C, and extension for 2 min at 72°C; after 30 cycles the reaction was additionally kept at 72°C for 7 min to complete extension. PCR products were purified with Wizard PCR prep DNA purification system (Promega corporation), and both strands of the PCR fragments were sequenced directly with an ABI Model 377 autosequencer using the Dye Terminator Ready Reaction Kit according to the manufacturer’s protocol (Perkin Elmer). The two amplification primers were also used for sequencing. Internal primers used for sequencing were as follows: B1F, 5′-CNCAYTNCTNCTGTTGAGRCG-3′; B2F, 5′-YGAYTGTGNCYGATGTG-3′; B1R, 5′-CGAYGNCKATRTKCTCATC-3′; and B2R, 5′-TTRAANGCRTTNACACCACCA-3′. The sequence of Ceratitis capitata was obtained from three separately amplified and cloned overlapping segments. Sequencing was done with the forward and reverse primers of the vector, otherwise using the procedures of method a. (A sequence of C. capitata is available from GenBank, Y08388, but it may have come from a different species. See Appendix 2.)

Sequence Analysis and Phylogeny Reconstruction. Sequences were entered, edited, and assembled using programs of the Fragment Assembly module and aligned using PILEUP and LINEUP of the GCG package (Version 9.1). Alignment required a 3-bp-long gap to be in-
serted in the same position (385–387 in Appendix 1) in all *Sophophora* sequences. The MEGA program (Kumar et al. 1993) was used to calculate distances and to construct evolutionary trees with the neighbor-joining (NJ) method (Saitou and Nei 1987), and for calculating several descriptive statistics. Maximum-parsimony trees were constructed using the PHYLIP 3.57 package (Felsenstein 1989). Alternative topologies were compared using Templeton (1983) and Kishino and Hasegawa (1989) tests, implemented in PAUP [Version 4.0.0d64 (Swofford 1998)]. Codon usage bias was measured with ENC (or $n_c$, the effective number of codons) (Wright 1990). Higher values of ENC indicate less codon usage bias.

Our phylogenetic analysis includes three additional genes: *Sod*, *Adh*, and *Gpdh* (except *D. busckii*). *Sod* and *Gpdh* were also analyzed for *D. virilis*, *D. immigrans*, and *D. mimica*.

### Results

The *Ddc* gene structure, amplification, and sequencing strategy are shown in Fig. 1. The 24 sequences (23 Drosophilidae plus the medfly *Ceratitis capitata*) are given in Appendix 1. Across the 23 Drosophilidae taxa, 413 sites are variable (43% of the 966 in the sequence; nt1:nt2:nt3, 79:34:300). Three hundred sixty-six sites are parsimony-informative (nt1:nt2:nt3, 61:24:275). The 10 species of the subgenus *Sophophora* lack a codon (nucleotide positions 385–387 in Appendix 1) that codes for asp in the other species. There is not much bias in GC

### Table 1. The 24 species studied

| Family     | Genus   | Subgenus | Group   | Species          | ID No. |
|------------|---------|----------|---------|------------------|--------|
| Drosophilidae | *Drosophila* | *Sophophora* | melanogaster | melanogaster | 1*     |
|            |         |          | simulans |                   | 2*     |
|            |         |          | teissier |                   | 3*     |
|            |         |          | erecta  |                   | 4*     |
|            |         |          | obscura | bifasciata        | 5*     |
|            |         |          |         | bogotana          | 6*     |
|            |         |          |         | persimilis        | 7*     |
|            |         |          | willistoni | paulistronum    | 8*     |
|            |         |          |         | willistoni        | 9*     |
|            |         |          |         | nebulosa          | 10*    |
|            |         |          | virilis | virilis           | 11*    |
|            |         |          | repleta | hydei             | 12*    |
|            |         |          | immigrans | immigrans      | 13*    |
|            |         |          |         | mimica            | 14*    |
|            |         |          | Scaptomyza | palmae           | 15*    |
|            |         |          |         | adusta            | 16*    |
|            |         |          | Hirtodrosophila | pictiventris  | 17*    |
|            |         |          | Doristophila | busckii       | 18*    |
|            |         |          | Zapronus | taberculatus      | 19*    |
|            |         |          | Liodrosophila | aerea         | 20*    |
|            |         |          | Samoaia | leonensis         | 21*    |
|            |         |          | Scaptodrosophila | lebanonensis | 22*    |
|            |         |          |         | amoena            | 23*    |
|            |         |          | Chymomyza | capitata        | 24*    |
| Tephritidae | *Ceratitis* | *Ceratitis* | *Ceratitis* | *Ceratitis* | *Ceratitis* |

* Asterisks indicate species analyzed for three additional genes: *Sod*, *Adh*, (except *D. busckii*), and *Gpdh* (except *D. adusta*, *D. mimica*, and *D. immigrans*).

* Modified mouthparts, a group of Hawaiian drosophilids.

* *Scaptodrosophila* is classified by Wheeler (1981) as a subgenus of *Drosophila* but has been raised to genus by Grimaldi (1990; see also Kwiatkowski et al. 1994, 1997). *Scaptomyza*, *Zaprionus*, *Liodrosophila*, and *Samoaia* are classified as genera by Wheeler (1981); in this paper we refer to them, as well as to *Hirtodrosophila* and *Doristophila*, as subgenera within the genus *Drosophila*.

![Fig. 1. Structure of the Ddc gene and strategy for amplification, cloning, and sequencing. The boxes represent exons; their coding parts are black and their noncoding parts are hatched. Thick lines represent the boxes are introns. The thick gray lines represent the segments amplified and sequenced, with primers shown as arrows above them. The sequence of *Ceratitis capitata* was obtained from three separately amplified fragments, which were cloned and both strands sequenced with standard vector primers. a and b refer to two methods; for the species studied by each method, see the text.](image-url)
content overall, but the variation is large at the third codon positions (Fig. 2), ranging from 0.47 in Chymomyza (even lower in Ceratitis, 0.41) to 0.77 in D. immigrans. The variation is notably large within the subgenus Sophophora, with the three willistoni group species having 52–61%, while the melanogaster and obscura groups have more than 70% GC. Codon usage bias, as expressed by the effective number of codons (ENC) does not differ among species (Fig. 2).

A neighbor-joining (NJ) tree based on Jukes–Cantor distances is presented in Fig. 3. Ceratitis (family Tephritidae) is used as the outgroup. A few species clusters are well resolved on the tree. All three species groups of the subgenus Sophophora form well-supported monophyletic groups, but the relationships among the three groups or between them and other Drosophilids are not well defined in this tree. The two species of Scaptomyza, one from Hawaii (D. palmae) and the other from Texas (D. adusta), form a monophyletic group that clusters in turn with the Hawaiian D. mimica with a high statistical (bootstrap) support. The two Drosophilidae genera, Chymomyza and Scaptodrosophila, are outside all other species, consistent with previous results (Kwiatowski et al. 1994, 1997), but with unreliable bootstrap values in the present case. Other NJ trees based on Kimura’s (1980) two-parameter distance and on the p-distance (proportion of different nucleotide sites) are consistent with Fig. 3 and yield similar statistically dependable relationships. A maximum-parsimony tree has a somewhat different topology, but with very low support for its nodes and yields monophyly for each of the Sophophora species groups, as well as for the association of Scaptomyza with D. mimica.

We have studied the same set of species (with the exceptions noted in Table 1) for three other genes (with the number of coding nucleotides analyzed, in parentheses): Sod (342), Adh (516), and Gpdh (729). For simplicity, only one species from each of the three Sophophora species groups is included in the analyses that follow, given that the monophyly of each group is so strongly supported in the Ddc tree (100% in each case) and otherwise. In the case of Adh we have replaced one species with another in two cases because of unavailability: the Scaptomyza species albovittata (rather than adusta) and Chymomyza procnesis (rather than amoena). Tamura et al. (1995) have studied Adh in numerous Scaptomyza species and concluded that they all form a monophyletic cluster.

Figure 4 displays four NJ trees based on Jukes–Cantor
distances obtained by considering other loci in addition to Ddc. Trees obtained with Kimura’s (1980) two-parameter or Tamura’s (1992) distance have precisely the same topologies as those shown in Fig. 4, and with similar bootstrap support. Maximum-parsimony trees also yield identical, or very similar topologies, but typically with lower bootstrap values than the NJ trees.

The combination of Ddc and Sod (Fig. 4A) brings bootstrap reliability to several nodes that were unresolved by Ddc alone. Incorporating also Adh (Fig. 4B) resolves most of the nodes of interest. Chymomyza and Scaptodrosophila are outside all other Drosophilids, with moderately strong indication that Chymomyza is the outgroup to the rest. The order of branching of these two genera has remained largely unresolved in the past. Throckmorton (1975) puts Scaptodrosophila in the ancestral position, while on Grimaldi’s (1990) tree their branching order was not resolved. Of the molecular studies that include both species, DeSalle (1992) considers Scaptodrosophila the most ancient, a position also favored by Kwiatowski et al. (1994, 1997), who point out the absence of statistical support for this hypothesis. Beverly and Wilson (1984) favored Chymomyza as the ancestral lineage. This ancestry of Chymomyza is also favored by combining Ddc + Sod + Gpdh (Fig. 4C), but with a low statistical reliability. The combination of all four genes (Fig. 4D) leaves the matter unresolved. If we use only codon positions 1 + 2, the NJ as well as the maximum-parsimony trees combining any three or all four genes place Scaptodrosophila as the outgroup to Chymomyza + Drosophila (Fig. 5).

Figure 4 shows the Sophophora subgenus (melanogaster, obscura, and willistoni groups) as the sister group to all other Drosophila, namely, the cluster of the Drosophila subgenus plus Scaptomyza, Hirtodrosophila, and Zaprinus (93% bootstrap value in Fig. 4B and 81% in
Fig. 4C, which includes, in addition, the subgenus Dor-silopha, but not D. immigrans or the cluster Scaptomyza + D. mimica. The position of the Sophophora subgenus as the outgroup to the other Drosophila subgenera (Drosophila, Hirtodrosophila, and Zaprimonus) is also firmly supported (97% bootstrap) by the combination of all four genes. The same conclusion is obtained if we use only codon positions1+2 and also reached by Tamura et al. (1995), based on the analysis of an Adh sequence longer than the one used in our analysis.

The conclusion that the subgenus Sophophora is monophyletic to all other Drosophila subgenera is supported by our observation of a 3-bp deletion (385–387 in Appendix 1) that appears in all Sophophora species (the three willistoni group species as well as in the obscura and melanogaster groups) but not in any of the other Drosophila subgenera (or in any of the outgroup genera, Scaptodrosophila, Chymomyza, and Ceratitis). The position of the willistoni group species based on genetic distances is equivocal, since the willistoni species often appear outside all other Drosophila lineages, including the other Sophophora (e.g., Pélandakis and Solignac 1993; Powell 1997), which may be a consequence of atypical molecular evolution in the willistoni group, as it is apparent in Fig. 2 with respect to third-position GC content. The monophyly of the Sophophora species is firmly supported when we analyze our data using only 1 + 2 codon positions.

All trees in Figs. 4 and 5 show D. virilis and D. hydei as a well-defined monophyletic cluster, as has also been determined in other molecular studies (Kwiatowski et al. 1994, 1997). The monophyly of D. mimica and Scaptomyza is also highly reliable (Figs. 4A and B, 5A), which is consistent with the Hawaiian origin of Scaptomyza, although this was classified as a separate genus by Wheeler (1981). Presumably, Scaptomyza shared with D. mimica a common ancestor within the Drosophila subgenus, in which D. mimica is usually included. The incorporation of Scaptomyza within the Drosophila subgenus is statistically supported in Figs. 4B and 5A by the association of the two pairs D. mimica + Scaptomyza and D. virilis + D. hydei (86 and 89% bootstrap, respectively). Nevertheless, the subgenus Drosophila would not seem to be monophyletic, even if we include Scaptomyza, because the species just mentioned appear to be equally or more closely related to the subgenus Hirtodrosophila than to other species of the subgenus Drosophila (D. immigrans; see below and Fig. 4A, B) when all sites are used. The subgenus Drosophila is not monophyletic either when the trees are based only on codon positions 1 + 2.

Figure 4 consistently shows Zaprimonus as the outgroup to all Drosophila subgenera, other than Sophophora, with a high statistical reliability in Fig. 4B (85% bootstrap) and Fig. 4D (92% bootstrap). However, when only codon positions 1 + 2 are used, the phylogenetic relationships are somewhat changed, so that Zaprimonus, Hirtodrosophila, and D. immigrans form a well-defined monophyletic group (82% bootstrap; Fig 5A). The reason for this discrepancy between the trees based on all positions or only 1 + 2 are not clear. One possibility could be differences in GC content in the third positions.

In order to address the problem of compositional bias, we have analyzed the data sets represented in Fig. 4 by
excluding species that are at the two opposite ends of the spectrum with respect to G₃ + C₃ content. In all sets, these species are *Ceratitis*, *Chymomyza*, *D. melanogaster*, and *D. bogotana*. (For *Ddc* + *Sod* we did the analyses with and without *D. immigrans*, which has high G₃ + C₃ in *Ddc*.) The differences in G₃ + C₃ content for the remaining species are small (≤10%), although these species represent all groups of interest. With this procedure the branching order remains the same as in Figs. 4A–C. Thus, GC content differences in the third codon positions do not seem to be the reason for the differences in branching order of *Zaprionus*, *Hirtodrosophila*, and *D. immigrans*, when based on different codon position sites. As we show below, the possible saturation at third position sites is not a factor either, because a plot of the divergences at 1 + 2 versus third positions clearly shows the absence of saturation, especially when *Ceratitis* is not used (as it has not been used in the above analysis).

The branching sequence of *Scaptodrosophila* and *Chymomyza* (Figs. 3 and 4A–C) becomes reversed if we exclude the third codon positions (Fig. 5). Is this a consequence of substitutional saturation at third positions? The evidence favors a negative answer. For the combined data set of four genes, third-position sites remain informative throughout the Drosophilidae and even for the more distant *Ceratitis*. Plots of the divergences of the Drosophilidae species at position 3 versus positions 1 + 2 do not indicate saturation at the third position for any gene or combination thereof (Fig. 6). Similarly, the number of differences at the third position is greater for the comparison between *Ceratitis* and any Drosophilidae species than for any comparisons between Drosophilidae (data not shown). We note here that a recent study by Yang (1998) shows that the bias, commonly attributed in the literature to saturation, may have been exaggerated. Simulations show that saturation occurs only at a much higher level of sequence divergence than has previously been suggested. Yang (1998) has pointed out that, by some current criteria, many data sets would be declared as saturated, even before enough substitutions have accumulated to be informative. According to Yang (1998), a much more serious problem than saturation is the absence of sufficient information at low levels of divergence.

Another potentially confounding effect may arise from differences in GC content in the third position (G₃ + C₃). Figure 6 shows the pairwise comparisons between all Drosophilidae species for third versus 1 + 2 positions. It is apparent that comparisons involving *Chymomyza* (squares in Fig. 6) generally show a relatively higher divergence at the third-position sites (Table 2). However, *Chymomyza* has the lowest G₃ + C₃ content of all Drosophilidae (see Fig. 2). The question is whether the higher divergence at the third position reflects an earlier split of *Chymomyza* from the other Drosophilidae or,
rather, the number of differences at the third position becomes inflated because of the lower incidence of G3 + C3 in *Chymomyza*. To the extent that this effect of nucleotide composition exists at all, it does not seem to be large, since we have found no correlation between the number of nucleotide differences and the differences in G3 + C3 content for all comparisons between *Chymomyza* and the *Drosophila* species (data not shown).

A more serious problem affecting phylogenetic inferences derives from the heterogeneity of substitution rates. Figure 6 shows that the number of substitutions between *Chymomyza* and the other species is relatively small with respect to positions 1 + 2, i.e., most squares are about midrange along the x axis, even though a majority of comparisons are between pairs of species more closely related to each other than they are to *Chymomyza*; the only partial exceptions are the comparisons with *Scaptodrosophila* (triangles in Fig. 6). This observation contrasts with the large number of substitutions in third positions, as already noted. The discrepancy is most extreme for *Adh*, but it is also clear for *Ddc* and the three genes combined. In Table 2 we show the average number of differences between species of the *Zapri- onus* clade (the seven top species in Fig. 4B) and each of five species ancestral to this clade. For two genes, *Ddc* and *Adh*, the number of substitutions at 1 + 2 positions is consistently smaller between *Chymomyza* and the species of the *Zapri- onus* clade than between the *Sophophora* species and the *Zapri- onus* clade. With respect to the third position, the opposite is the case; at both *Ddc* and *Adh*, the number of substitutions is consistently greater for the comparisons with *Chymomyza* than with the *Sophophora* species. A similar but much reduced discrepancy occurs for the comparisons with *Scaptodrosophila*. With respect to *Sod*, however, the number of differences at positions 1 + 2 is somewhat greater in the comparisons involving *Chymomyza* and *Scaptodrosophila*, as expected; but at the third positions, the *Sophophora* species are as different from *Chymomyza* and *Scaptodrosophila* as from the *Zapri- onus* clade. The conclusion of this analysis is that the rates of nucleotide substitutions, as reflected in the comparison of 1 + 2 versus third position, are variable according to patterns that are inconsistent from gene to gene and from lineage to lineage. This is likely to impact phylogenetic inferences based on numbers of nucleotide substitutions. We may add that, with respect to the number of amino acid replacements in *Gpdh*, there seems to have occurred a rapid acceleration in the *Chymomyza* lineage (Ayala et al. 1996; Kwiatowski et al. 1997), which is just the opposite of the pattern we have just noted for *Ddc* and *Adh*. In any case and for the time being, it seems safe to conclude that the branching order of *Scaptodrosophila* and *Chymomyza* relative to *Drosophila* remains unresolved, although our analysis favors somewhat the hypothesis that the *Chymomyza* lineage is older than *Scaptodrosophila*.

Figure 7 displays six trees with 12 *Drosophilidae* taxa (and *Ceratitis* as the outgroup). We have tested them statistically, using the combined data for *Ddc*, *Adh*, and *Sod*, by the methods of Templeton (1983) and Kishino and Hasegawa (1989), both of which yield qualitatively identical results. Table 3 gives the results of the Kishino–Hasegawa tests, which have been performed for the same trees, using all sites or only codon position sites 1 + 2. Tree 1 is favored by our analysis of all sites (the same topology as Fig. 4B). Tree 2 differs from tree 1 only in the position of *Chymomyza* and *Scaptodrosophila*. Tree 3 is favored by the analysis of positions 1 + 2. Trees 2 and 3 are statistically not worse than tree 1 when we use all sites. Trees 4, 5, and 6 represent, respectively, the phylogenetic hypotheses of Throckmorton (1975), Grimaldi (1990), and DeSalle (1992a,b, 1995). Every one of trees 4, 5, and 6 is statistically worse than tree 1, if based on all sites. When 1 + 2 positions are used, tree 3 is statistically preferred over all others, except tree 2.

Figure 8 displays trees that include the subgenus *Dorsiloph* and that are tested using data for only two genes, *Ddc* and *Sod*. Tree 1 has the same topology as tree 1 in Fig. 7 (and Fig. 4B), but with the inclusion of *Dorsiloph* between *Zapri- onus* and *D. immigrans*, as favored by our data (Fig. 4A). Tree 2 is the phylogeny favored by analysis of 1 + 2 codon position sites. Trees 3 and 4 correspond, respectively, to the phylogenetic hypotheses of Throckmorton (1975) and Grimaldi (1990). Trees 1 and

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**Table 2. Average number of nucleotide substitutions (mean ± SE) between the listed species and the *Zapri- onus* clade at each of the genes *Ddc*, *Sod*, and *Adh* for different codon positions**

| Gene | 1 + 2 | 3rd |
|------|-------|-----|
| *Chymomyza* | 27.9 ± 1.9 | 166.1 ± 3.6 |
| *Scaptodrosophila* | 36.1 ± 1.7 | 153.0 ± 2.3 |
| *D. melanogaster* | 47.3 ± 2.3 | 137.1 ± 3.5 |
| *D. bogotana* | 38.4 ± 1.8 | 147.0 ± 3.7 |
| *D. willistoni* | 37.6 ± 1.9 | 144.4 ± 3.2 |

* The *Zapri- onus* clade includes *D. virilis*, *D. hydei*, *D. (Scaptomyza)* adusta, *D. mimica*, *D. pictiventris*, *D. immigrans*, and *D. (Zapri- onus)* tuberculatus.
2 do not differ statistically from each other, whether all positions or only positions 1 + 2 are used; trees 3 and 4 are statistically inferior to trees 1 and 2 (Table 4).

We have also compared trees that are based on all four loci (shown in Figs. 4D and 5B). These trees do not differ statistically from each other by the Kishino–Hasegawa test, whether all positions or only positions 1 + 2 are used. Trees that correspond to the hypotheses of Throckmorton (1975), Grimaldi (1990), and DeSalle (1992a, b, 1995) are statistically worse in both cases than those in Figs. 4D and 5B.

Discussion

A potential benefit of combining data from several loci when testing phylogenetic hypotheses is that the phylogenetic signal weakly present in some genes becomes amplified (Baker and DeSalle 1997). The combined analysis of the three nuclear genes, Ddc, Adh, and Sod, produces the tree shown in Fig. 4B (see also tree 1 in Fig. 7), which has the same topology (but with more taxa included) as the tree obtained by adding a fourth gene, Gpdh (Fig. 4D), if all sites are used. Separate analysis of the combined data for Ddc and Sod allows us to incorporate the subgenus Dorsilopha (D. busckii) in that tree (Fig. 4A and tree 1 in Fig. 8). Use of only positions 1 + 2 yields trees (Fig. 5) that are largely congruent with those obtained when all sites are used. It is not clear, however, which set of trees should be given preference. While positions 1 + 2 are less prone to the effect of saturation and nucleotide-composition bias than third positions, they are more likely to be under selective constraints, and this could impact the phylogenetic analysis.
Our analysis shows some heterogeneity between all sites and positions 1 + 2 in the Drosophilid lineages, particularly with respect to *Chymomyza*. We have noted that the effects of saturation and nucleotide-composition bias do not seem to be detectable at the third positions. This suggests that trees based on all sites may be most informative. Nevertheless, it is most conservative to consider the position of *Chymomyza* relative to *Scaptodrosophila* as unresolved, especially considering that the Kishino–Hasegawa (1989) and Templeton (1989) tests show that trees based either on all positions or only positions 1 + 2 do not differ statistically. A consensus tree based on all analyses is shown in Fig. 9.

Consistent topologies are obtained and are well supported when pairs of the four genes we have studied are analyzed, although few alternatives become resolved in the separate analysis of individual genes. The combination of data from different genes has to be made with the awareness, as we have shown, that rates of evolution vary among taxa in patterns that are different from gene to gene, and even within a gene, as observed when comparing codon positions 1 + 2 versus 3 (see Results). The
Two issues are at stake: (1) whether the Sophophora subgenus is monophyletic and (2) whether Sophophora is an outgroup to the other Drosophila subgenera (and some nominal genera), namely, Zaprionus, Scaptomyza, Hirtodrosophila, Dorsilopha, and the subgenus Drosophila, including the Hawaiian Drosophila.

Traditional taxonomies consider the subgenus Sophophora to be a monophyletic taxon that embraces the willistoni, melanogaster, and obscura groups, as well as other groups not included in our study (Wheeler 1981; Patterson and Stone 1952). Several molecular analyses, however, place the willistoni group outside a clade that includes all other Drosophila, although typically with a low statistical confidence (e.g., Pelandakis and Solignac 1993; Kwiatowski et al. 1994, Figs. 3A and B; Kwiatowski et al. 1997, Fig. 3). This willistoni group position as the sister clade to all other Drosophila, including the set of the other Sophophora groups, such as melanogaster and obscura, may be considered correct but it may also be attributed to distinctive characteristics of the molecular evolution of the willistoni group, such as an accelerated rate of nucleotide substitutions and low G3 + C3 content (review by Powell 1997). The NJ Ddc tree shown in Fig. 2 places the willistoni group within the Sophophora clade, but with a low bootstrap value. Nevertheless, when the Ddc data are combined with Sod alone, or also with Adh and Gpdh, the monophyly of the Sophophora subgenus is statistically well supported (Fig. 4). This is also the case when only positions 1 + 2 are taken into account (Fig. 5). Moreover, the Ddc gene sequences (Appendix 1) provide unambiguous evidence that Sophophora is a monophyletic subgenus, because there is a deletion of three coding nucleotides (sites 385–387 in Appendix 1) shared by all Sophophora species but no other Drosophilid species (or by Ceratitis). Our results also provide strong support to the traditional interpretation that places Sophophora within the genus Drosophila (in the sensu latto we use), but as the first Drosophila clade to branch off, and thus as the sister group to all other Drosophila subgenera, as proposed by Throckmorton (1975). A majority of molecular studies supports this positioning of Sophophora (Thomas and Hunt 1993; Kwiatowski et al. 1994, 1997; Tamura 1995) (see Table 5). Our analysis of Ddc indicates, again in accordance with Throckmorton (1975), that other groups

![Consensus tree resulting from combined analyses of four nuclear genes, based on all sites as well as on codon positions 1 + 2. All resolved nodes are strongly supported.](image)

### Table 4. Kishino–Hasegawa test of the four tree topologies shown in Fig. 8, using the combined data for Ddc and Sod with either all codon position sites or only positions 1 + 2: Differences are in comparison to the best treea

| Tree | Length | Length difference ± SD | p   | Tree | Length | Length difference ± SD | p   |
|------|--------|------------------------|-----|------|--------|------------------------|-----|
| 1    | 2337   | Best                    | —   | 1    | 505    | 6 ± 6                  | 0.32|
| 2    | 2349   | 12 ± 13                 | 0.34| 2    | 499    | Best                   | —   |
| 3    | 2404   | 67 ± 15                 | <0.0001| 3    | 518    | 19 ± 6                 | <0.001|
| 4    | 2408   | 71 ± 14                 | <0.0001| 4    | 528    | 29 ± 9                 | <0.001|

a Trees 1 and 2 represent phylogenetic hypotheses favored by analysis of all sites and by analysis of 1 + 2 position sites, respectively; trees 3 and 4 represent, respectively, the phylogenetic hypotheses of Throckmorton (1975) and Grimaldi (1990). The test of Templeton (1983) yields qualitatively identical results.
also are in a derived position relative to *Sophophora* (e.g., the genera *Samoai* and *Liodrosophila*). Although data have been available for years indicating that *Sophophora* is an early-diverged lineage [e.g., *Sod* (Kwiątowski et al. 1994) and *Adh* (Tamura et al. 1995)], other authors have recently favored the hypothesis placing the *Sophophora* lineage closer to other *Drosophila* subgenera than *Zaprionus* and *Hirtodrosophila* (DeSalle 1995; Powell 1997; Powel and DeSalle 1995). Our analysis of the combined data for four genes (Figs. 4 and 5), as well as the recent analysis of Remsen and DeSalle (1998), clearly contradicts this hypothesis. The suggestion that *Zaprionus* is “a good choice” as an outgroup to the genus *Drosophila* (Powell 1997, pp. 275–276) can hardly be maintained.

Our analysis does not agree, however, with Throckmorton’s claims concerning the branching order among the rest of the *Drosophila* species, which make up the whole sister clade to *Sophophora*. Throckmorton divides the rest of the species considered here into two clades: the “*virilis–repleta* lineage,” which includes *D. hydei* and *D. virilis*, and the “*immigrans–Hirtodrosophila* lineage,” which includes *D. immigrans*, *Zaprionus*, *Scaptomyza*, *Hirtodrosophila*, *Dorsilopha*, and the Hawaiian *Drosophila*. Tamura et al. (1995), based on analysis of *Adh*, have suggested that the Hawaiian groups of *Drosophila* and *Scaptomyza* form a monophyletic group, which is closest to the species in the *virilis–repleta* lineage, but are not included in the *immigrans–Hirtodrosophila* lineage. Our analysis of the combined data for four genes (which include *Adh*) supports Tamura and co-workers’ (1995) proposal. The monophyly of *Scaptomyza* and the Hawaiian *Drosophila* is favored by virtually all molecular studies. Placing these two groups as the sister clade to the *virilis–repleta* set contradicts DeSalle’s (1992, 1995) conclusion, based on mtDNA, that the Hawaiian flies are an early offshoot of the subgenus *Drosophila*. But it agrees with the recent conclusion of Remsen and DeSalle (1998), based on the combined analysis of several genes.

Our results show that the subgenus *Drosophila* (represented in our study by *D. virilis*, *D. repleta*, *D. mimicus*, and *D. immigrans*) is likely to be paraphyletic (see Fig. 4, trees A–C, and Fig. 5A), although this is not definite in the consensus tree (Fig. 9), with respect to the genus *Drosophila*. Kwiątowski et al. (1997) suggested removing some paraphyly by downgrading the status of the genus *Zaprionus* to the subgeneric level. But if one is to retain *Sophophora* as a *Drosophila* subgenus, it becomes necessary by cladistic rules also to downgrate *Scaptomyza* and, possibly, the genera *Liodrosophila* and *Samoai*. When this is done, *Drosophila* is not only a genus “with too many species,” but also a genus “with too many subgenera.”

An alternative possibility would be to raise *Sophophora* to the rank of genus. This would seem justified by the old age of *Sophophora*, which diverged from the other *Drosophila* no less than 50 million years ago (and by the old age of the divergence between the *willistoni* and the *melanogaster* groups, which is no less than 40 million years old) and also by the existence of several hundred *Sophophora* species. However, it is unrealistic to expect that thousands of *Drosophila* geneticists would accept this proposal and refer henceforward to *D. mela-

### Table 5.
Position of the genera and subgenera listed in the left column with regard to the subgenera *Sophophora* and *Drosophila* according to various molecular studies

| Taxon                      | Ancestral to *Sophophora* + *Drosophila* (Grimaldi’s hypothesis) | Placed with s.g. *Drosophila*, while *Sophophora* is outgroup (Throckmorton’s hypothesis) |
|---------------------------|---------------------------------------------------------------|------------------------------------------------------------------------------------------|
| *Liodrosophila*           | mtDNA (DeSalle 1992a)                                        | *Adh* (Thomas and Hunt 1993; Russo et al. 1995)                                          |
| *Zaprionus*               | mtDNA (DeSalle 1992a)                                        | *Adh* (Thomas and Hunt 1993; Russo et al. 1995)                                          |
| *Samoai*                  |                                                               | *Gpdh* (Kwiątowski et al. 1997)                                                          |
| *Dorsilopha*              |                                                               | *Sod* (Kwiątowski et al. 1994)                                                           |
| *Engioscaptomyza*         |                                                               | *18SRNA* (Pelandakis and Solignac 1993)                                                  |
| *Scaptomyza*              |                                                               | *Gpdh* (Thomas and Hunt 1993; Russo et al. 1995)                                         |
| *Hirtodrosophila*         | mtDNA (DeSalle 1992a)                                        | *LHP* (Beverly and Wilson 1984)                                                          |
| *Hawaiian Drosophila* (Idiomya) |                                                               | *mtDNA* (DeSalle 1992b)                                                                 |

Also in the consensus tree (Fig. 9), with respect to the genus *Drosophila*. Kwiątowski et al. (1997) suggested removing some paraphyly by downgrading the status of the genus *Zaprionus* to the subgeneric level. But if one is to retain *Sophophora* as a *Drosophila* subgenus, it becomes necessary by cladistic rules also to downgrate *Scaptomyza* and, possibly, the genera *Liodrosophila* and *Samoai*. When this is done, *Drosophila* is not only a genus "with too many species,” but also a genus “with too many subgenera.”

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nogaster as *Sophophora melanogaster* in the thousands of papers published each year that deal with *D. melanogaster*. Rather more sensible, as a matter of practice, is to enlarge the genus *Drosophila*, as done in Table 1, so that it embraces several taxa formerly ranked as genera.

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Appendix 1

Fig. A1. Twenty-five sequences of Ddc. The sequence Y08388 is from Mantzouridis et al. (1997).
Fig. A1. Continued.
| Species                | DNA Sequence 1 | DNA Sequence 2 | DNA Sequence 3 | DNA Sequence 4 | DNA Sequence 5 | DNA Sequence 6 |
|------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| melanogaster simulans | AAGCAAGTCAGCA AGTGTGGCTC TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG |
| teissieri              | TACCCCTCAGT CAGTACACG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG |
| erecta                 | TACCCCTCAGT CAGTACACG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG |
| bifasciata             | TACCCCTCAGT CAGTACACG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG |
| bogotana               | TACCCCTCAGT CAGTACACG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG |
| persimilis             | TACCCCTCAGT CAGTACACG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG |
| paulistorum            | TACCCCTCAGT CAGTACACG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG |
| willistoni             | TACCCCTCAGT CAGTACACG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG |
| nebulosa               | TACCCCTCAGT CAGTACACG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG TCTTTAGGCT CAGTACAGG |

Fig. A1. Continued.
Appendix 2

T.D. Mantzouridis, D.C. Sideris, and E.G. Fragulis (Gene 204:85–89, 1997) have published a cDNA *Ddc* sequence attributed to the medfly *Ceratitis capitata*. Figure A1, bottom row, gives the alignment of this sequence, Y08388, with the others reported in this paper. Figure A2 gives the position of Y08388 in a simplified NJ tree. It is apparent from this tree (and Appendix 1) that Y08388 represents a gene sequence that has only recently (within the last 2–5 million years) diverged from *D. melanogaster*. We have also compared Y08388 with sequences from the related *amd* (α-methyl dopa-hypersensitive) gene, which is assumed to have arisen with *Ddc* from an ancient duplication event. The *amd* genes from *D. melanogaster* (Marsh et al. 1986), as well as from several Drosophilids sequenced in our laboratory, are all extremely distant from any of the *Ddc* genes. Indeed, the fruitfly *amd* gene is more remotely related to any fruitfly *Ddc* gene than any of these is to human *Ddc*. It seems likely that Y08388 comes from a species closely related to *D. melanogaster* rather than from *Ceratitis capitata*. A possible alternative explanation is that Y08388 represents a second *Ceratitis Ddc* gene, acquired by lateral transfer from one of the *melanogaster*-group species within the last 2–5 million years. The transfer of a functional gene between two animals has no known precedent, and it must be therefore considered very unlikely.