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Ddc and *amd* Sequences Resolve Phylogenetic Relationships of *Drosophila*

With about 3000 species, the family Drosophilidae is large and diverse even by Dipteran standards. This diversity provides biologists with distinctive opportunities to investigate evolutionary patterns, but also poses taxonomic and other challenges. Thus, the traditional classification (e.g., Wheeler, 1981) is inconsistent with phylogenetic relationships based on morphology (Throckmorton, 1975; Grimaldi, 1990) or molecular data (review in Powell, 1997). The two comprehensive phylogenetic hypotheses of Throckmorton (1975) and Grimaldi (1990) have been tested against recent molecular studies, which have resolved some important discrepancies between them (see Remsen and DeSalle, 1998; Tatarenkov *et al.*, 1999a; Kwiatowski and Ayala, 1999; Katoh *et al.*, 2000). Yet, many phylogenetic relationships remain unsolved, such as those among *Hirtodrosophila*, *Zaprionus*, *Dorsilopha*, and s.g. *Drosophila* (e.g., Tatarenkov *et al.*, 1999a; Kwiatowski and Ayala, 1999). Some genera, such as *Liodrosophila* and *Samoaia*, have received scarce attention (Pélandakis and Solignac, 1993; Tamura *et al.*, 1995; Tatarenkov *et al.*, 1999a) and their phylogenetic placement remains largely unknown. One problem with the molecular phylogenies is the incompleteness of taxa sampling. Although representatives of some *Drosophila* groups have been included together in some studies, often different studies include different groups, which prevents construction of a reliable higher-level phylogenetic framework.

We seek to define a robust framework of relationships in the Drosophilidae at the species-group and higher taxonomic levels. We have investigated 29 species (Table 1) from several drosophilid genera and subgenera and from representative species groups for two nuclear genes, dopa decarboxylase (*Ddc*) and α-methyl dopa (*amd*). These are closely linked paralogous genes, arisen by an ancient gene duplication (Eveleth and Marsh, 1986; Tatarenkov *et al.*, 1999b). We earlier used *Ddc* to address some issues of *Drosophila* systematics (Tatarenkov *et al.*, 1999a). We now extend our previous investigation by including additional taxa for longer sequences of *Ddc* and a new gene, *amd*, which previously had been sequenced only in *D. melanogaster* (Marsh *et al.*, 1986), *D. simulans*, and *Scaptodrosophila lebanonensis* (Tatarenkov *et al.*, 1999b).

DNA preparation and sequencing were as described by Tatarenkov *et al.* (1999a; method b). The 963- to 966-bp-long sequences of *Ddc* previously reported are now extended to 1131–1134 bp. *Ddc* sequences of five more species, *D. pseudoobscura*, *D. robusta*, *D. phalerata*, *D. funebris*, and *D. gymnobasis*, are added. Amplification and sequencing of *amd* was as for *Ddc*, except that the annealing temperature was 60°C and the extension time was 3 min. The *amd* amplifying primers were 5′-MAYATGCAYGSCTAYTAYCCCAC-CAG-3′ (Amd-un2, forward primer) and 5′-ACCA-TRTAGATYTTYTTNCGNTCCAT-3′ (Amd-bw, reverse primer). The amplified region of *amd* encompasses an intron. The amplified fragment varied in length from 1269 bp in *D. hydei* to nearly 2600 bp in *D. tripunctata*, depending on the length of the intron. Only the coding regions, 1032-bp-long, were used in this study (66 bp

from exon 1 and 966 bp from exon 2), because the large divergence of intron sequences made their alignment impossible. Internal primers for sequencing were Amd1: 5′-GNACNTGYGCNTAYGAYGA-3′; Amd1-Rev: 5′-GCATCNACRTGNARCCASAC-3′; Amd2: 5′-GTNGTNATGGAYTGGYTGG-3′; and Amd2-Rev: 5′-GTGCANGCNGGRCTRCADAT-3′. We succeeded in amplifying only a short fragment (927 bp) from the second exon of *Liodrosophila aerea* using Amd2 and Amd-bw. Sequences were aligned with programs PILEUP and LINEUP of the GCG package (Wisconsin package, Version 9.1). The alignment required that a 3-bp-long gap be inserted at positions 433–435 in all *Sophophora Ddc* sequences. Phylogenetic analyses were performed with PAUP (version 4.0b1 for Macintosh; Swofford, 1998) and MEGA (Kumar *et al.*, 1993). We present trees from only the neighbor-joining (NJ) analyses, but all data sets were also analyzed with maximum-parsimony (MP), minimum-evolution (ME), and maximum-likelihood (ML, HKY-gamma substitution model) methods. We use the incongruence length difference (ILD) test (Farris *et al.*, 1994), called the partition-homogeneity test in PAUP, to evaluate incongruence between data partitions. Invariant characters were removed before the ILD test was applied.

Because of the shorter sequence of *amd* (927 versus 1032 bp) for *L. aerea*, we conducted all analyses twice, using a set of shorter *amd* sequences that included *L. aerea* and a longer set without it. The trees based on these two sets are congruent, thus allowing for straightforward interpretations.

Similarly to other previously studied genes, the *amd* sequences show considerable variation in nucleotide composition at the 3rd codon position. Strong nucleotide compositional bias, coupled with high divergence levels (maximum values of raw sequence divergence are 25% for *Ddc* and 29% for *amd*) raise the issue of whether the 3rd codon positions remain informative for phylogenetic reconstruction. We have exploited the fact that *amd* and *Ddc* are ancient paralogous genes (the duplication preceded the divergence of Diptera–Lepidoptera, under the molecular clock assumption). Nucleotide composition varies in similar fashion among species at both genes. If only 3rd codon positions are used, we expect the sequences of both genes to intermingle in the phylogeny if these positions are not informative. Instead, an NJ tree based on only 3rd codon position shows two clusters corresponding to *amd* and *Ddc* (100% bootstrap). This indicates that the 3rd codon positions are informative. Additional evidence in favor of the use of 3rd codon positions comes from ILD tests showing that there is no incongruence among the three codon positions, for each gene separately or for their combination.

The results of the separate analyses of *amd* and *Ddc* are fully encompassed in their combined analysis and, therefore, are not presented. We will mention only that

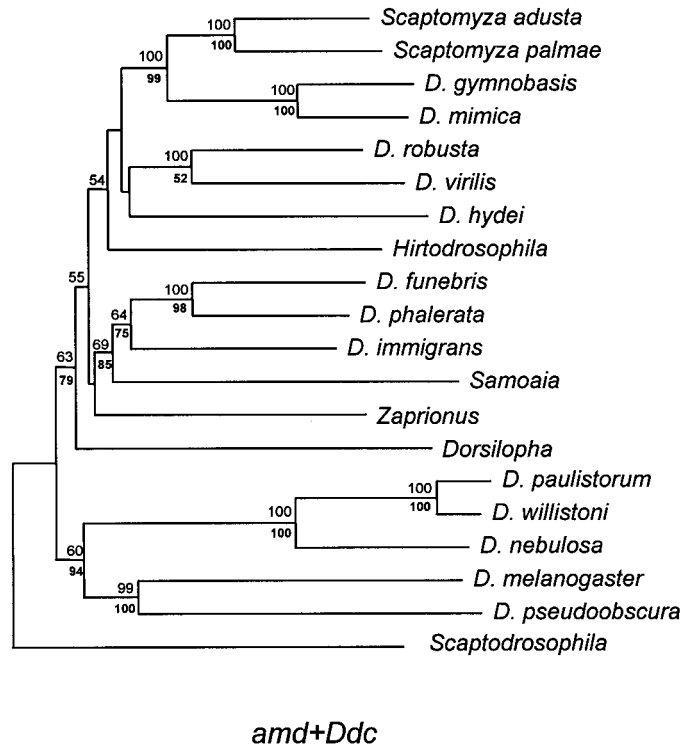

FIG. 1. NJ tree based on Kimura's two-parameter distances using the 2163-bp-long combined sequences of *amd* (1032 bp) and *Ddc* (1131 bp). The tree is obtained with all codon positions; bootstrap confidence values are above branches. Bootstrap values obtained with 1st + 2nd codon positions are shown below branches. *Liodrosophila* is not included in the tree because the coding sequence obtained is only 927 bp long (rather than 1032 bp) for *amd*. If we use only the homologous 927 bp for all species (plus 1131 bp of *Ddc*; total 2058 bp), *Liodrosophila* clusters with *Zaprionus*; the tree topology remains the same but the bootstrap support becomes less for some nodes.

in the NJ analysis of *amd*, *D. tripunctata* and *D. putrida*, which were not studied for *Ddc*, cluster with *D. funebris* and *D. phalerata* with strong bootstrap support of 98%; the relationships among all four species are not resolved. *D. immigrans* is their closest sister taxon (bootstap 71%). Each of the three species groups of *Sophophora* is strongly defined on the *amd* tree (bootstrap 100%). Taking into account the well-established monophyly of these groups, we obtained longer *Ddc* sequences for only one species from each of the *melanogaster* and *obscura* groups of the subgenus *Sophophora*, but three species of the *willistoni* group, because the 3rd codon nucleotide composition in species of the *willistoni* group is rather distinct from that of the other *Drosophila*.

The *amd* and *Ddc* data partitions are not incongruent, according to the ILD tests (Farris *et al.*, 1994), whether based on all nucleotides or on the 1st + 2nd codon positions only. The NJ tree based on the analysis of all *amd*+*Ddc* nucleotides is presented in Fig. 1, with bootstrap values above branches for all codon positions and below branches for 1st + 2nd codon positions. The

TABLE 1

The 29 Species Studied

Genus	Subgenus	Group	Species[a]	GenBank Accession No. *amd*	*Ddc*	*Adh*
Drosophila	*Sophophora*	*melanogaster*	*melanogaster**	X04695	AF091328	X78384
			simulans	AF293726		
			teissieri	AF293727		
			erecta	AF293708		
		obscura	*bifasciata*	AF293705		
			bogotana	AF293706		
			persimilis	AF293720		
			*pseudoobscura**	AF293722	AF293746	X62181
		willistoni	*paulistorum**	AF293719	AF293744	AB026529
			*willistoni**	AF293730	AF293750	L08648
			*nebulosa**	AF293717	AF293742	DNU95275
	Drosophila	*virilis*	*virilis**	AF293729	AF293749	AB033640
		robusta	*robusta*	AF293724	AF293747	
			sordidula			AB033639
		repleta	*hydei**	AF293712	AF293737	X58694
		immigrans	*immigrans**	AF293713	AF293738	M97638
		testacea	*putrida*	AF293723		
		quinaria	*phalerata*	AF293721	AF293745	
			brachinephros			AB033644
		funebris	*funebris*	AF293709	AF293734	AB033643
		tripunctata	*tripunctata*	AF293728		
		HPW[c]	*gymnobasisi*	AF293710	AF293735	
			hawaiiensis			DHU48715
		MMP[c]	*mimica**	AF293716	AF293741	M60792
	Scaptomyza[b]		*palmae*	AF293718	AF293743	AB033649–AB033651
			*adusta**	AF293704	AF293732	
	Hirtodrosophila		*pictiventris**	AF293711	AF293736	AB026530
	Dorsilopha		*busckii**	AF293707	AF293733	
	Zaprionus[b]		*tuberculatus**	AF293731	AF293751	X63955
	Liodrosophila[b]		*aerea*	AF293715	AF293740	AB033655–AB033657
	Samoaia[b]		*leonensis*	AF293725	AF293748	
Scaptodrosophila[b]			*lebanonensis*	AF293714	AF293739	M97637

Note. Newly obtained *amd* and *Ddc* sequences are underlined. All *Ddc* sequences have been extended from 966 bp (Tatarenkov *et al.*, 1999a) to 1134 bp.

[a] Asterisks indicate species analyzed for *Sod*; GenBank accession numbers are given in Kwiatowski and Ayala (1999).

[b] *Scaptodrosophila* is classified by Wheeler (1981) as a subgenus of *Drosophila*, but has been raised to genus by Grimaldi (1990). *Scaptomyza, Zaprionus, Liodrosophila*, and *Samoaia* are classified as genera by Wheeler (1981); in this paper we shall refer to them, and to *Hirtodrosophila* and *Dorsilopha*, as subgenera within the genus *Drosophila*.

[c] Hawaiian picture-winged (HPW) and modified mouth parts (MMP) are groups of Hawaiian drosophilids.

first split is between the subgenus *Sophophora* and all other species. In the tree based on the 1st + 2nd codon positions both clades are strongly supported, whereas support is only moderate when all positions are used. These two major clades also emerge on the maximum-parsimony tree based on the 1st + 2nd codon positions, although with somewhat lesser support than that on the corresponding NJ tree.

The Hawaiian drosophila and *Scaptomyza* are monophyletic. The well-defined pair *virilis/robusta*, together with *hydei*, appear as the closest sister taxa to the Hawaiian/*Scaptomyza* in all analyses, but without strong support. *Samoaia, D. immigrans, D. phalerata*, and *D. funebris* form a well-outlined group (85% based on 1st + 2nd codon positions, 69% based on all nucleotides). Relationships among the previously noted clusters and the other taxa are not well resolved.

To obtain better-resolved phylogenies, we used two additional genes, *Adh* and *Sod*, which have been sequenced in many of the species that we have studied (Table 1). For *Adh* we use *D. sordidula* rather than *D. robusta, D. brachinephros* rather than *D. phalerata*, and *D. hawaiiensis* rather than *D. gymnobasis*. For *Sod* we use *D. guttifera* rather than *D. phalerata*. We have conducted separate analyses for each gene, combined analyses, and examined the data for heterogeneity with the ILD test. According to this test, the phylogenetic signals present in each of the genes that we have investigated are largely congruent. The general conclusion is that whereas some genes can be particu-

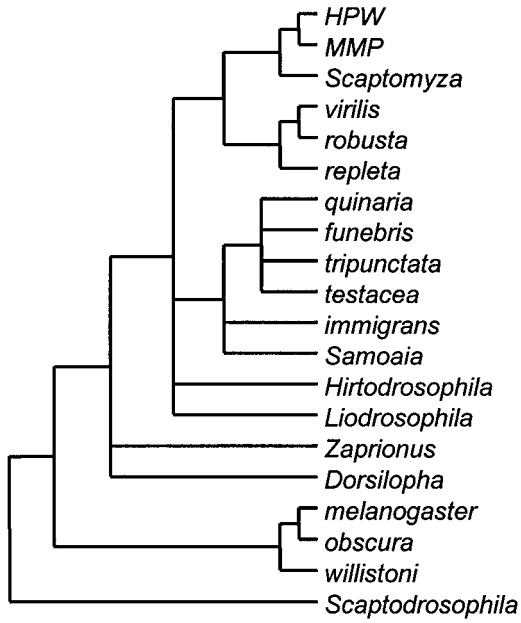

FIG. 2. Summary tree of the phylogenetic relationships among the subgenera and species groups of the genus *Drosophila*. This tree is an amalgam of nonconflicting results obtained from separate and combined analyses of four genes, *amd*, *Ddc*, *Adh*, and *Sod*. Hawaiian picture-winged (HPW) and modified mouth parts (MMP) are groups of Hawaiian drosophilids.

larly informative about certain nodes on the tree, the best resolution is obtained when all genes are combined. In no case did we observe that significant bootstrap support (≥75%) for a clade based on a single gene decreased after other genes for which support for that clade was not high were added.

Figure 2 summarizes our results. Not all species shown on the tree were studied at each gene, but it is possible to incorporate them in the consensus tree because of general agreement between the single gene and the combined analyses. A brief discussion of certain clades on the tree follows. We concentrate mostly on the results of *amd*, *Ddc*, and *Adh* analyses because they include most species.

According to Throckmorton (1975), *Sophophora* is placed at the base of the *Drosophila* phylogeny. But in Grimaldi's (1990) cladogram *Sophophora* is a sister group to the subgenus *Drosophila* (which is narrowly defined by exclusion of the Hawaiian *Drosophila*, genus *Idiomyia* sensu Grimaldi). Moreover, some earlier molecular studies (Pélandakis and Solignac, 1993) have suggested that the *willistoni* and *saltans* groups may not be monophyletic with the *melanogaster* and *obscura* groups. In our separate analysis of the genes, there is moderate support for the early branching (76%) and monophyly (70%) of *Sophophora* when the *Adh* data are used, whereas *amd* and *Ddc* are only weakly informative. However, pooling of *amd* and *Ddc* brings more resolution: bootstrap support for the monophyly of *Sophophora* increases to 72%, and support for its early split from the rest of the drosophilids becomes 66%. The combined analysis of the three genes yields strong support for the early branching (95%) and monophyly (85%) of *Sophophora*. Addition of *Sod* further increases bootstrap values to 98 and 89%. Moreover, the monophyly of *Sophophora* is strongly supported by one codon deletion in *Ddc* in all *Sophophora* species (Tatarenkov *et al.*, 1999a).

Similarly, the position of *Zaprionus* as the outgroup to all non-*Sophophora* species becomes strongly supported on the combined trees, whereas its position is not definite on single gene trees.

Some clusters of species receive high bootstrap support in each of the separate analyses. In the NJ analysis of *amd*, there is strong support (bootstrap 98%) for the clade comprising *D. putrida* (*testacea* group), *D. funebris* (*funebris*), *D. phalerata* (*quinaria*), and *D. tripunctata* (*tripunctata*). *Ddc* and *Adh* were not studied in species of the *testacea* and *tripunctata* groups, but *funebris* and representatives of the *quinaria* group cluster together with very high support for each gene (100 and 99%). As expected, the cluster of *D. funebris* and *D. phalerata/brachinephros* is strongly supported in the combined analysis of the three genes. This finding is significant because it resolves the previously uncertain position of *D. funebris*. Throckmorton (1975) places it at the base of the *Drosophila* radiation. According to his scheme, after the split of *Sophophora*, the *funebris* group was among the first lineages (the other is the genus *Liodrosophila*) to separate from the rest of *Drosophila*. But according to Grimaldi (1990), the *funebris* group occupies a more derived position on the tree within the subgenus *Drosophila*. *D. immigrans* appears as the sister group to the cluster of species just discussed, in the analysis of *Adh* (56%) and *amd* (71%). When sequences from the three genes are combined, bootstrap support for the cluster comprising *D. immigrans*, *D. phalerata/brachinephros*, and *D. funebris* becomes 98%. Grouping of *D. immigrans* with the *quinaria*, *tripunctata*, and *funebris* groups is also suggested by the 28S rRNA gene (Pélandakis and Solignac, 1993), although without statistical support (<50%).

The separate analysis of the three genes yields in all cases a clade comprising *Scaptomyza* and the Hawaiian *Drosophila* (represented here by the picture-winged and modified-mouth-parts groups), with support which is strong in *Ddc* (92%), moderate in *amd* (78%), and weak in *Adh* (65%). When the three genes are combined, this cluster receives support of 99%. Throckmorton (1975) considered the Hawaiian groups of *Drosophila* and *Scaptomyza* sister taxa and monophyletic with respect to other *Drosophila* groups, whereas Grimaldi (1990) proposed that the Hawaiian *Drosophila* together with *Hirtodrosophila* represent an early offshoot in the subfamily Drosophilinae and

raised the Hawaiian *Drosophila* to generic status (*Idiomyia*). All molecular data indicate that *Scaptomyza* is the closest sister taxon to the Hawaiian *Drosophila*.

An unexpected early result from molecular studies was that the closest sister group to the cluster *Scaptomyza*/Hawaiian *Drosophila* was the *virilis–repleta* lineage [shown in the analysis of *Adh* by Russo *et al.* (1995) and Tamura *et al.* (1995)]. How do other genes support this hypothesis? Separate analyses of *amd* and *Ddc* are not informative on this matter, but in the combined analysis of these genes, the Hawaiian Drosophilids do cluster together with the *virilis–repleta* groups with weak support, and when *Sod* is added the support becomes 77% (*Sod* separately supports this cluster only weakly, at 22%). It thus appears that the clade Hawaiian *Drosophila/Scaptomyza* is indeed the sister taxon to the *virilis–repleta* lineage. Although the strongest evidence for this comes from *Adh*, the total evidence from three other genes also supports it. When *amd*, *Ddc*, and *Adh* are pooled, the cluster of the Hawaiian drosophilids and the *virilis–repleta* lineage achieves bootstrap support of 96%.

The positions of *Liodrosophila* (not studied for *Sod*), *Hirtodrosophila*, and *Dorsilopha* (not studied for *Adh*) are not definite and require additional study. Analysis of *amd*, *Ddc*, and *Adh* clearly indicates that *Liodrosophila* and *Hirtodrosophila* are derived with regard to *Zaprionus*. On the other hand, in the combined analysis of *amd*, *Ddc*, and *Sod*, *Hirtodrosophila*, together with other non-*Sophophora* species, appears in a derived position with regard to both *Dorsilopha* and *Zaprionus*. Tentative relationships among these taxa are shown in Fig. 2.

No single gene has yet produced an unequivocal phylogeny of the Drosophilidae. Instead, the pooling together of data sets from several genes seems promising. The analysis of new *amd* and *Ddc* sequences from all major radiations (sensu Throckmorton, 1975), together with the previously studied *Adh* and *Sod*, provides a reasonably detailed resolution of phylogenetic relationships of Drosophilidae (Fig. 2) that may serve as a working hypothesis in future studies.

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