J Mol Evol (1993) 37:525–543

Journal of Molecular Evolution

Molecular Phylogeny of *Drosophila* Based on Ribosomal RNA Sequences

Michel Pélandakis, Michel Solignac

Laboratoire de Biologie et Génétique évolutives, C.N.R.S., 91198 Gif-sur-Yvette Cedex, France

Received: 22 June 1992 / Revised and accepted: 5 February 1993

Abstract. Nucleotide sequences of 72 species of Drosophilidae were determined for divergent D1 and D2 domains (representing 200 and 341 nucleotides respectively in *D. melanogaster*) of large ribosomal RNA, using the rRNA direct sequencing method. Molecular phylogenetic trees were reconstructed using both distance and parsimony methods and the robustness of the nodes was evaluated by the bootstrap procedure. The trees obtained by these methods revealed four main lineages or clades which do not correspond to the taxonomical hierarchy. In our results, the genus *Chymomyza* is associated with the subgenus *Scaptodrosophila* of the genus *Drosophila* and their cluster constitutes the most ancient clade. The two other clades are constituted of groups belonging to the subgenus *Sophophora* of the genus *Drosophila:* the so-called Neotropical clade including the *willistoni* and *saltans* groups and the *obscura-melanogaster* clade itself split into three lineages: (1) *obscura* group + *ananassae* subgroup, (2) *montium* subgroup, and (3) *melanogaster* + Oriental subgroups. The fourth clade, the *Drosophila* one, contains three lineages. *D. polychaeta, D. iri,* and *D. fraburu* are branched together and constitute the most ancient lineage; the second lineage includes the *annulimana, bromeliae, dreyfusi, melanica, mesophragmatica, repleta, robusta,* and *virilis* groups. The third lineage is composed of the *immigrans* and the *cardini, funebris, guaramunu, guarani, histrio, pallidipennis, quinaria,* and *tripunctata* groups. The genera *Samoaia, Scaptomyza,* and *Zaprionus* are branched within the *Drosophila* clade. Although these four clades appear regularly in almost all tree calculations, additional sequencing will be necessary to determine their precise relationships.

Key words: *Drosophila — Zaprionus — Phylogeny — Ribosomal RNA sequences*

Correspondence to: M. Pélandakis

The genus *Drosophila* has diversified into more than 1,500 species (Ashburner 1989). This is probably not attributable to an unusual high rate of speciation but, more likely, to the antiquity of the genus, which is more than 50 Myr old (Throckmorton 1975; Beverley and Wilson 1984). Morphological traits generally show high uniformity whereas other factors, including genetic and ecological characters, have undergone substantial evolutionary divergence. However, the paucity of phylogenetic inferences derived from morphological analyses at the level of major clades within the genus *Drosophila* comes more from the lack of appropriate studies than from the lack of useful morphological characters. Noticeable exceptions are the classical works of Throckmorton (1975) and more recently the studies of Okada (1989) and Grimaldi (1990), who have reconsidered the taxonomical framework and produced phylogenetic trees based on morphological characters in the family Drosophilidae. Several molecular techniques have also been used to elucidate the phylogenetic relationships among the species of the genus *Drosophila:* immunoprecipitation of the larval hemolymph protein (Beverley and Wilson 1982, 1984); 2D gel electrophoresis of proteins (Spicer 1988); sequencing of ADH (Sullivan et al.

1990), nuclear rRNA (Pélandakis et al. 1991), and mtDNA (DeSalle 1992); and DNA-DNA hybridization (Caccone et al. 1992). Recently, DeSalle and Grimaldi (1991) published a review which emphasized the importance of molecular analyses for systematics and compared the results of morphological and molecular methods.

In order to investigate the phylogenetic relationships within the genus *Drosophila* and related genera, we have chosen to sequence two domains of the large subunit (28S) of nuclear ribosomal RNA. This molecule, even if it is not a perfect chronometer, has proved to be a powerful tool in studying the phylogeny of distantly related taxa by comparison of the sequences of the so-called conserved core of the molecule. Interspersed with the regions of the conserved core there are 12 divergent domains (D1–D12, Hassouna et al. 1984). Preliminary analysis has shown that the only region variable enough to study species of the genus *Drosophila* was the D2 domain and, to a lesser extent, the D1 domain. The sequences of these two domains have been established for 72 Drosophilidae and several outgroups. This allowed us to infer the phylogenetic relationships of species of the genus *Drosophila* and related genera. They are branched in four clades, which exhibit some interesting differences compared to classical taxonomy.

Materials and Methods

Drosophila strains. Seventy-two species belonging to the Drosophilidae family were analyzed, 71 Drosophilinae and one Steganinae. The majority of the strains used were cultured in the Laboratoire de Biologie et Génétique Evolutives in Gif-sur-Yvette; the others were obtained from Bowling Green University. Various species belonging to different dipteran families were used as outgroups: *Myatropa florea* (Syrphidae), *Glossina tachinoides* (Glossinidae), *Ceratitis capitata* (Tephritidae), *Calliphora vomitaria* (Calliphoridae), and *Delia radicum* (Anthomyiidae).

I. Genus *Drosophila*.

A. Subgenus *Dorsilopha: D. busckii.*

B. Subgenus *Drosophila:* annulimana group: *D. aracatacas, D. gibberosa, D. talamancana;* bromeliae group: *D. bromeliae;* cardini group: *D. arawakana;* dreyfusi group: *D. camargoi;* funebris group: *D. funebris;* guaramunu group: *D. guaramunu;* guarani group: *D. guarani;* histrio group: *D. sternopleuralis;* immigrans group: *immigrans* subgroup: *D. immigrans; hypocausta* subgroup: *D. rubida;* melanica group: *D. melanica;* mesophragmatica group: *D. gaucha;* pallidipennis group: *D. pallidipennis;* polychaeta group: *D. polychaeta;* quinaria group: *D. phalerata;* repleta group: *hydei* subgroup: *D. hydei; mulleri* subgroup: *D. buzzatii; repleta* subgroup: *D. repleta;* robusta group: *D. robusta;* tripunctata group: *D. mediopictoides;* virilis group: *D. virilis;* ungrouped species: *D. iri, D. fraburu.*

C. Subgenus *Lordiphosa: D. andalousiaca.*

D. Subgenus *Scaptodrosophila:* coracina group: *D. dimorpha;* latifasciaeformis group: *D. latifasciaeformis;* victoria group: *D. deflexa, D. lebanonensis* and *D. rufifrons.*

E. Subgenus *Sophophora:* fima group: *D. fima;* melanogaster group: *ananassae* subgroup: *D. ananassae, D. malerkotliana, D. vallismaia, D. varians; elegans* subgroup: *D. elegans; eugracilis* subgroup: *D. eugracilis; ficusphila* subgroup: *D. ficusphila; melanogaster* subgroup: *D. erecta, D. mauritiana, D. melanogaster, D. orena, D. sechellia, D. simulans, D. teissieri, D. yakuba; montium* subgroup: *D. bakoue, D. kikkawai, D. malagassya, D. serrata; suzukii* subgroup: *D. mimetica; takahashii* subgroup: *D. takahashii;* obscura group: *affinis* subgroup: *D. affinis, D. azteca; obscura* subgroup: *D. pseudoobscura;* saltans group: *cordata* subgroup: *D. neocordata; elliptica* subgroup: *D. emarginata; saltans* subgroup: *D. prosaltans; sturtevanti* subgroup: *D. sturtevanti;* willistoni group: *D. nebulosa, D. willistoni.*

II. Genus *Zaprionus*.

A. Subgenus *Zaprionus: Z. capensis, Z. inermis, Z. taronus, Z. sepsoides.*

B. Subgenus *Anaprionus: Z. lineosus.*

III. Other genera: *Chymomyza bicolor, Samoaia leonensis, Scaptomyza pallida.*

RNA Extraction. Total RNA was prepared from adult flies. About 200 mg of material was homogoneised at 0°C in 2 ml of the extraction buffer (Tris 1 M pH 7.4; EDTA 0.1 M; SDS 5%). Proteins were removed by three to five phenol-chloroform-isoamyl alcohol (50–48–2) extractions. Nucleic acids were recovered through ethanol precipitation, and RNA was recovered by precipitation with 3 M LiCl (Maccecchini et al. 1979).

Sequencing Strategy. The rRNA sequences of all diptera analyzed were obtained by the direct method of sequencing (Qu et al. 1983). This method uses rRNA as a template, the reverse transcriptase as polymerase, and the dideoxy chain termination method of Sanger et al. (1977). Two variable regions, D1 and D2, of the 28S ribosomal RNA gene were sequenced. They are positioned between nucleotides 3372–3546 and 3705–4050, respectively, in the *D. melanogaster* sequence within the coordinates of Tautz et al. (1988). Primers complementary to evolutionary conserved segments adjacent to these variable domains were used, with the following sequences: 5′TGCATTCCCAAGCAACCCGACTCC3′ and 5′CCTTGGTCCGTGTTTCAAGACGGG3′, respectively, for the D1 and D2 domains. The primers were end-labeled with gamma^{32}P-dATP prior to the sequence reaction.

Sequence Alignment. Alignment of the cDNA sequences was carried out automatically with the CLUSTAL programs (Higgins and Sharp 1988) and subsequently checked with the help of secondary structures (Michot and Bachellerie 1987; Rousset et al. 1991), which are more conservative than primary ones. A hypervariable region in the D2 domain positioned from 3821 to 3829 in the 28S rRNA of *D. melanogaster* (coordinates of Tautz et al. 1988) was omitted from the calculation.

Phylogenetic Inferences. Molecular distances were estimated either by the ratio of nucleotide differences to the length of the sequences (*p* distance) or corrected by the K_{nuc} of Kimura (1980) (*k* distance). In order to use the information included in deletions/additions, the following strategy was adopted: For the *p* distance each nucleotide deleted or added was considered as a substitution; for the *k* distance, each nucleotide deleted or added was weighted as a transversion.

Phylogenetic trees were reconstructed by the distance matrix method using the neighbor-joining (NJ) algorithm (Saitou and Nei 1987). We also used three parsimony methods: DNAPARS, DNACOMP (Felsenstein's program PHYLIP package, version 3.01), and PAUP (Swofford 1990). The bootstrap procedure (Felsenstein 1985) was used to establish the score of each node (DNABOOT in Felsenstein's PHYLIP package; Jean-Marie Cornuet's SNJBOOT program).

Results

The 72 sequences of Drosophilidae and the sequence of *Delia radicum* obtained with the direct sequencing method are given in Fig. 1. They are aligned against the *D. melanogaster* sequence comprising 200 and 341 nucleotides for D1 and D2 domains respectively. They are presented with a total length of 201 and 362 nucleotides to include the additional nucleotides occurring at various positions in the different species. A hypervariable loop of the D2 domain, up to 10 bases long, difficult to sequence and not useful in this study, has not been taken into account in the calculations. Among the remaining nucleotides, 204 nucleotide sites were variable (37 for the D1 and 167 for the D2) and 127 were phylogenetically informative (20 and 107). A simplified matrix of the pairwise *p* distance is given in Table 1 for 39 selected species. The general phylogenetic tree calculated from the sequences by the NJ algorithm is given in Fig. 2.

Phylogenetic Relationships at the Generic and Subgeneric Levels

The sample encompassing all the drosophilins sequenced included 71 species: 63 belonging to the genus *Drosophila* and 8 belonging to related genera—i.e., *Chymomyza bicolor, Samoaia leonensis, Scaptomyza pallida,* and 5 species of the genus *Zaprionus*. Two additional species, *Delia radicum* (Anthomyidae) and *Leucophenga maculata* (Steganinae, Drosophilidae), were used as outgroups for rooting the general tree. Species belonging to distant families such as *Myatropa florea, Glossina tachinoides,* or *Ceratitis capitata* were used to confirm the external position of *Delia radicum* and *Leucophenga maculata* in the drosophilin tree.

With the total set of species, we only used the NJ algorithm. Several trees were calculated using single or multiple outgroups and either the *p* or the *k* distance. In all cases, the overall tree topology was divided into four main clusters, hereafter referred to as clades, which do not correspond to well-defined divisions in the taxonomical hierarchy.

The *Scaptodrosophila* clade encompasses the genus *Chymomyza* and the subgenus *Scapdodrosophila* of the genus *Drosophila*. The Neotropical clade is comprised of the *willistoni* and *prosaltans* groups of the subgenus *Sophophora*. The third clade—namely, the *obscura-melanogaster* clade—includes the remaining species studied in the subgenus *Sophophora,* i.e., the 25 species of the two major taxa, the *obscura* and *melanogaster* groups, and a minor one, the *fima* group. The *Drosophila* clade includes species belonging to the subgenera *Drosophila* and *Dorsilopha* of the genus *Drosophila* and species belonging to the genera *Zaprionus, Samoaia,* and *Scaptomyza*. The subgenus *Lordiphosa* of the genus *Drosophila* is placed as the sister group of the clade *obscura-melanogaster* or as the sister group of the *Drosophila* clade.

These results have been supported when the general sample was reduced to sets of 10–20 species which contained species representative of the four clades previously revealed in the general trees. With these reduced samples, both parsimony and distance (NJ) methods were used to produce phylogenies. The phylogenetic trees which emerged from these analyses comprised the same four main clades as in the general tree, with the exception of the clade *obscura-melanogaster,* the monophyly of which is not always supported by the parsimony methods. The same is true for the *Scaptodrosophila* clade due to the position of the genus *Chymomyza*. The bootstrap test applied to the NJ trees indicates a relatively low score for these clades (smaller than 40%) whereas each of the two other clades (the Drosophila and Neotropical clades) had a score of at least 80%. The subgenus *Scaptodrosophila* is supported by 75% of the bootstrap replicates.

If the determination of these four clades was relatively clear, it was in turn difficult to obtain precise relationships between them. With the whole sample, the NJ method gave two alternative topologies presented in Fig. 3, depending on the outgroups or the type of calculations. In both trees, the *obscura-melanogaster* clade branched with the *Drosophila* clade but, in one case, the Neotropical and *Scaptodrosophila* clades shared a direct common ancestor whereas in the other one the Neotropical clade was associated with the *obscura-melanogaster/Drosophila* cluster. The stability of the phylogenetic relationships was not significantly improved when the subsamples were analyzed. The resulting phylogenetic trees gave different topologies de-

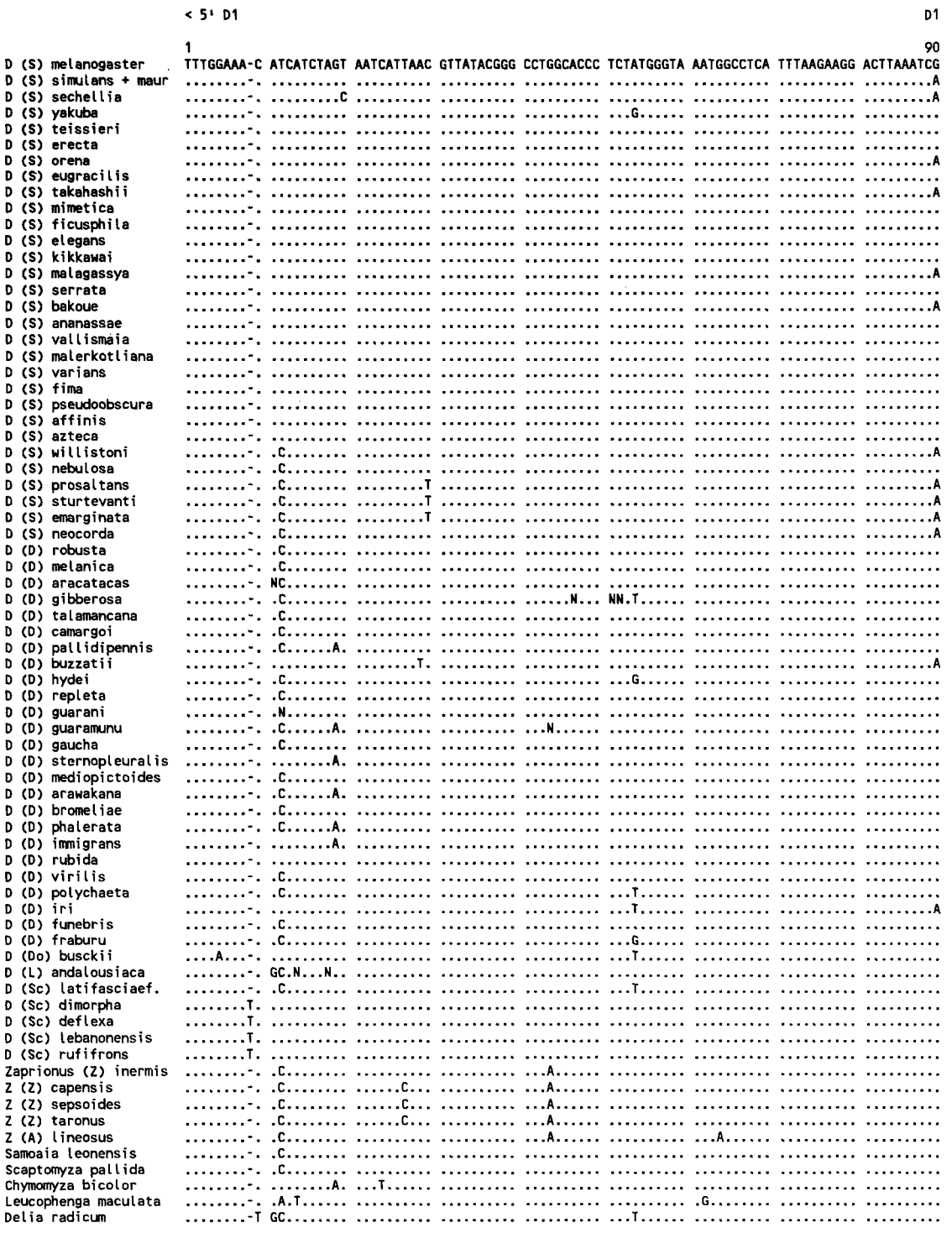

Fig. 1. Aligned sequences of 72 Drosophilidae species and of *Delia radicum*. The sequence of *D. mauritiana* (maur) was identical to that of *D. simulans*. *Dashes* denote gaps in the aligned sequences. Nucleotides ambiguous on the gels (nonspecific arrests) are indicated by *N*. Segments corresponding to the D1 and D2 domains are indicated above the figure. The sequence of the hypervariable loop (*hvl*) was not determined for most species. Continued on pages 529–533.

pending on the species considered. However, the Neotropical clade was rarely found as the sister group of the *obscura-melanogaster* clade.

Several sites (always the same) exhibited multiple hits whatever the topology of the tree. We have tried to discard the most variable among them. Sites exhibiting 10 or more and then 8 or more substitutions in most tree topologies were successively removed (respectively, 11 and 18 sites). The four major clades continued to emerge but the stability of the phylogenetic relationships between them was not strengthened. The bootstrap values were not

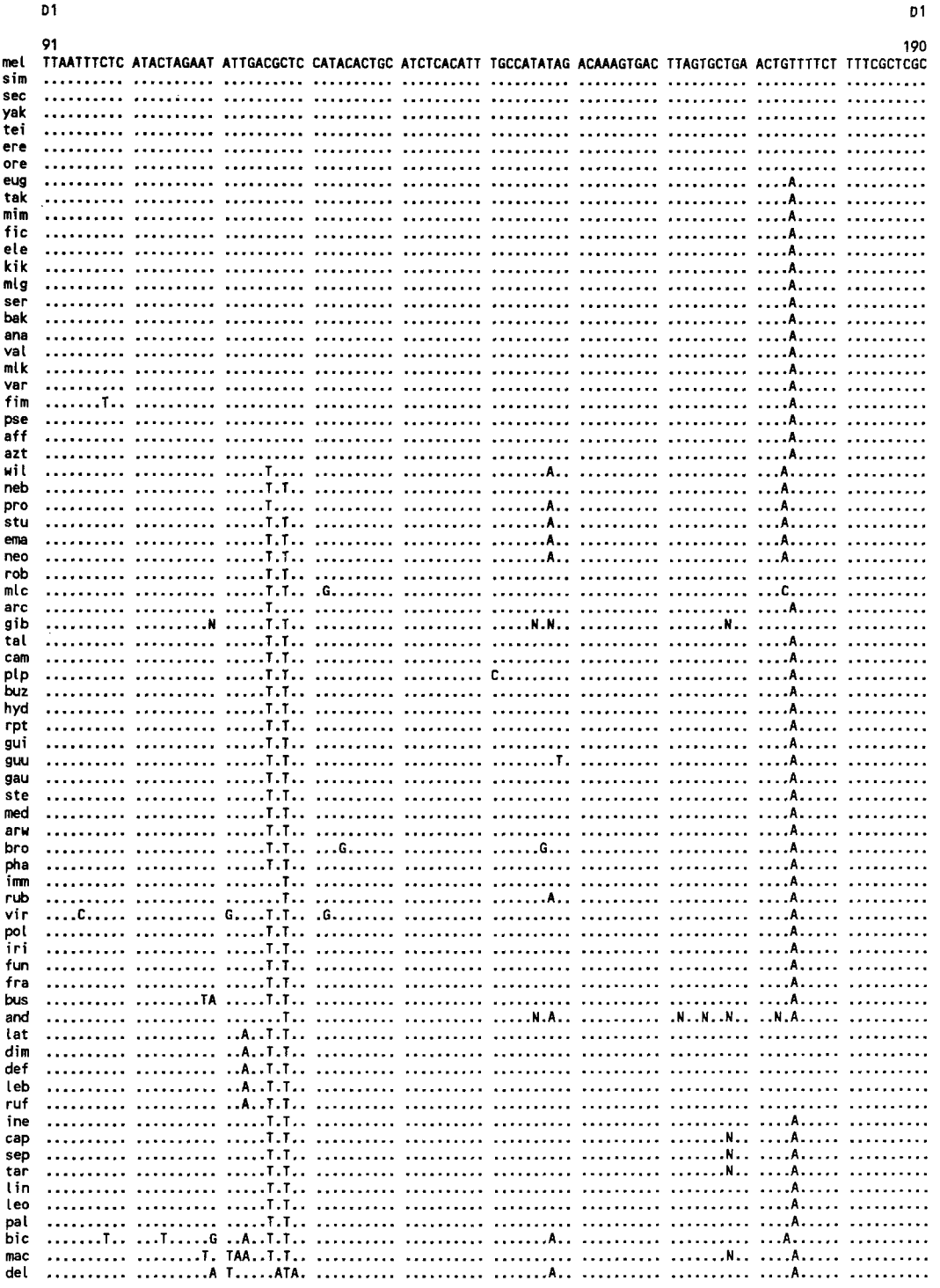

Fig. 1. Continued.

increased when the hypervariable sites were discarded except for that of the *obscura-melanogaster* clade.

The Scaptodrosophila *Clade*

The *Scaptodrosophila* clade includes all the species analyzed belonging to the subgenus *Scaptodrosophila* (*coracina* species group, *victoria* species group, *latifasciaeformis* species group) and *Chymomyza bicolor,* the species representative of the large genus *Chymomyza*. This clade is split into three lineages. The first consists of the *coracina* and *victoria* species groups, i.e., *D. dimorpha, D. rufifrons, D. lebanonensis,* and *D. deflexa*. The second corresponds to *D. latifasciaeformis* and the third to *Chy-*

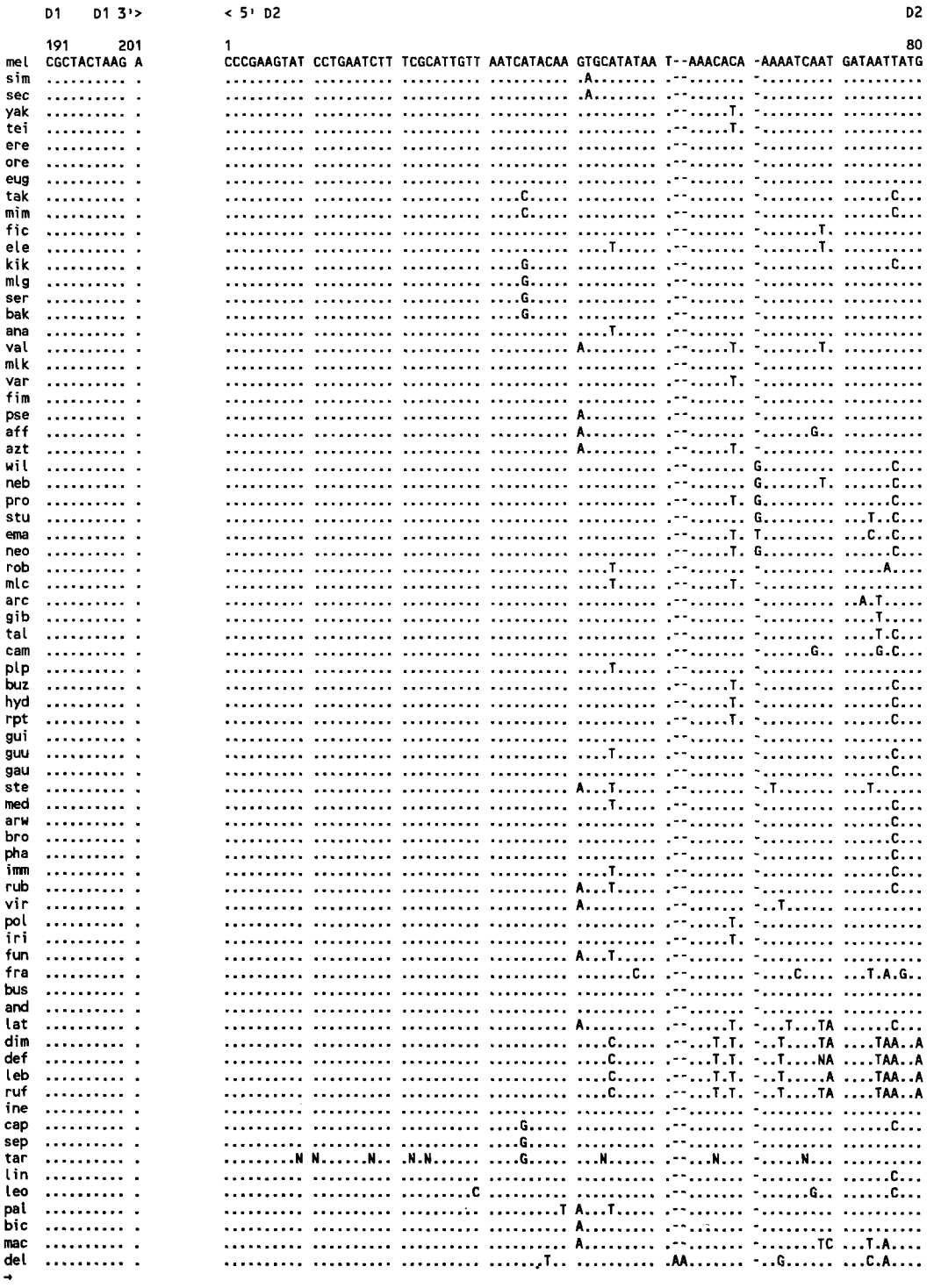

Fig. 1. Continued.

momyza bicolor. In our trees, *Chymomyza bicolor* is either the sister group of the species of the subgenus *Scaptodrosophila* as depicted in Fig. 4 or the sister group of the *victoria/coracina* groups.

The Neotropical and the Sophophora *Clades*

These two clades have already been analyzed in a previous study (Pélandakis et al. 1991).

The *saltans* and the *willistoni* groups are always clustered together in the so-called Neotropical clade. Species of the *saltans* group constitute a very homogeneous cluster which is branched within the species of the *willistoni* group.

The *Sophophora* clade comprises three main lineages: the first includes the *melanogaster* and the so-called Oriental subgroups; the second the *mon-*

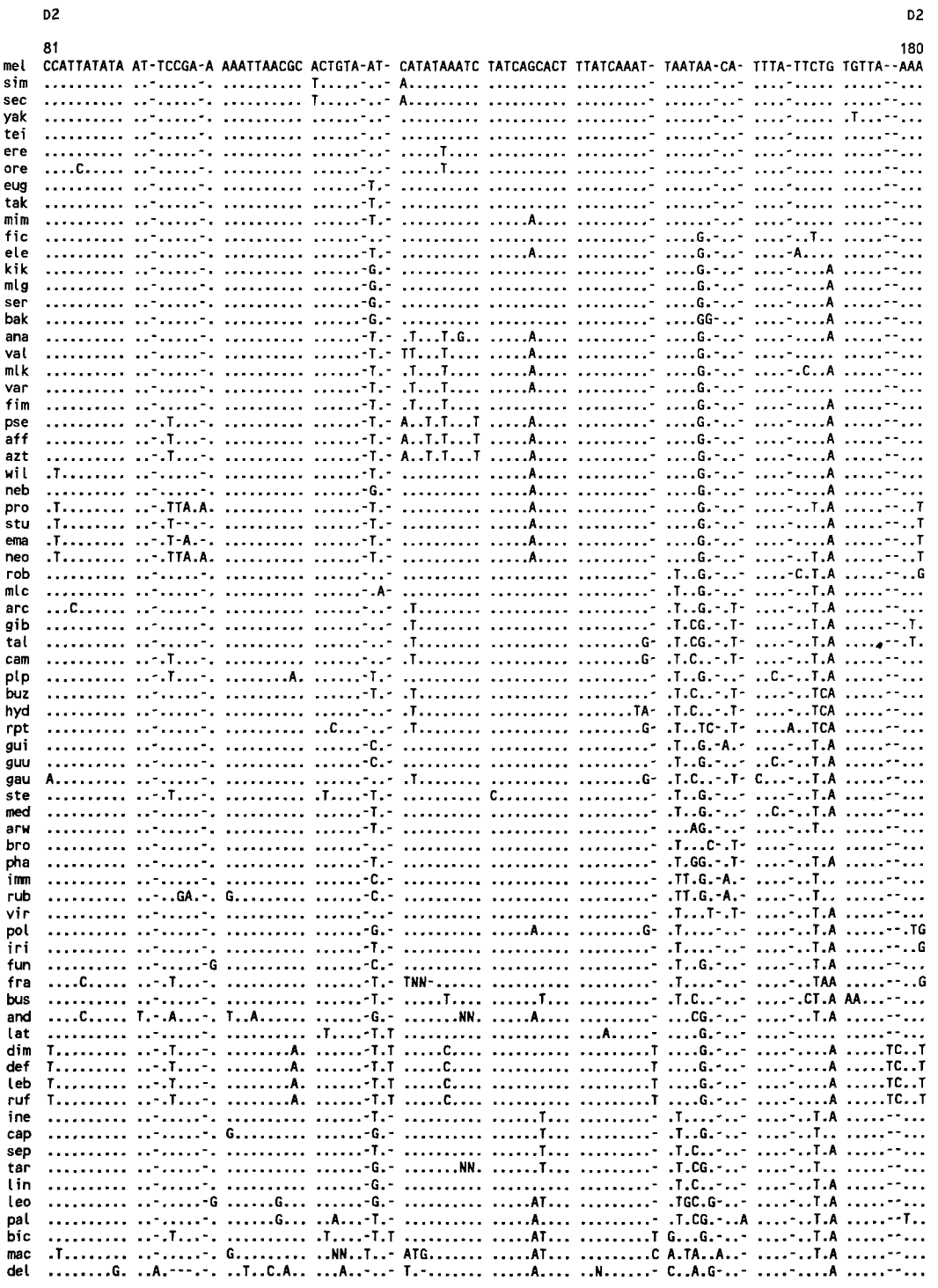

Fig. 1. Continued.

tium subgroup; and the third the *ananassae* subgroup (all the subgroups previously listed belong to the *melanogaster* group), which was found associated within the species of the *obscura* and *fima* groups. Whatever the method used, these three lineages emerged but the trichotomy at the basis of these three lineages remains unsolved, the nodes being very close.

The Drosophila *Clade*

The phylogenetic tree obtained for the *Drosophila* clade is given in Fig. 5. This clade comprises the genera *Samoaia, Scaptomyza,* and *Zaprionus,* the subgenus *Dorsilopha,* and all the species of the subgenus *Drosophila*. The topology deduced from the parsimony method (program DNAPARS) was es-

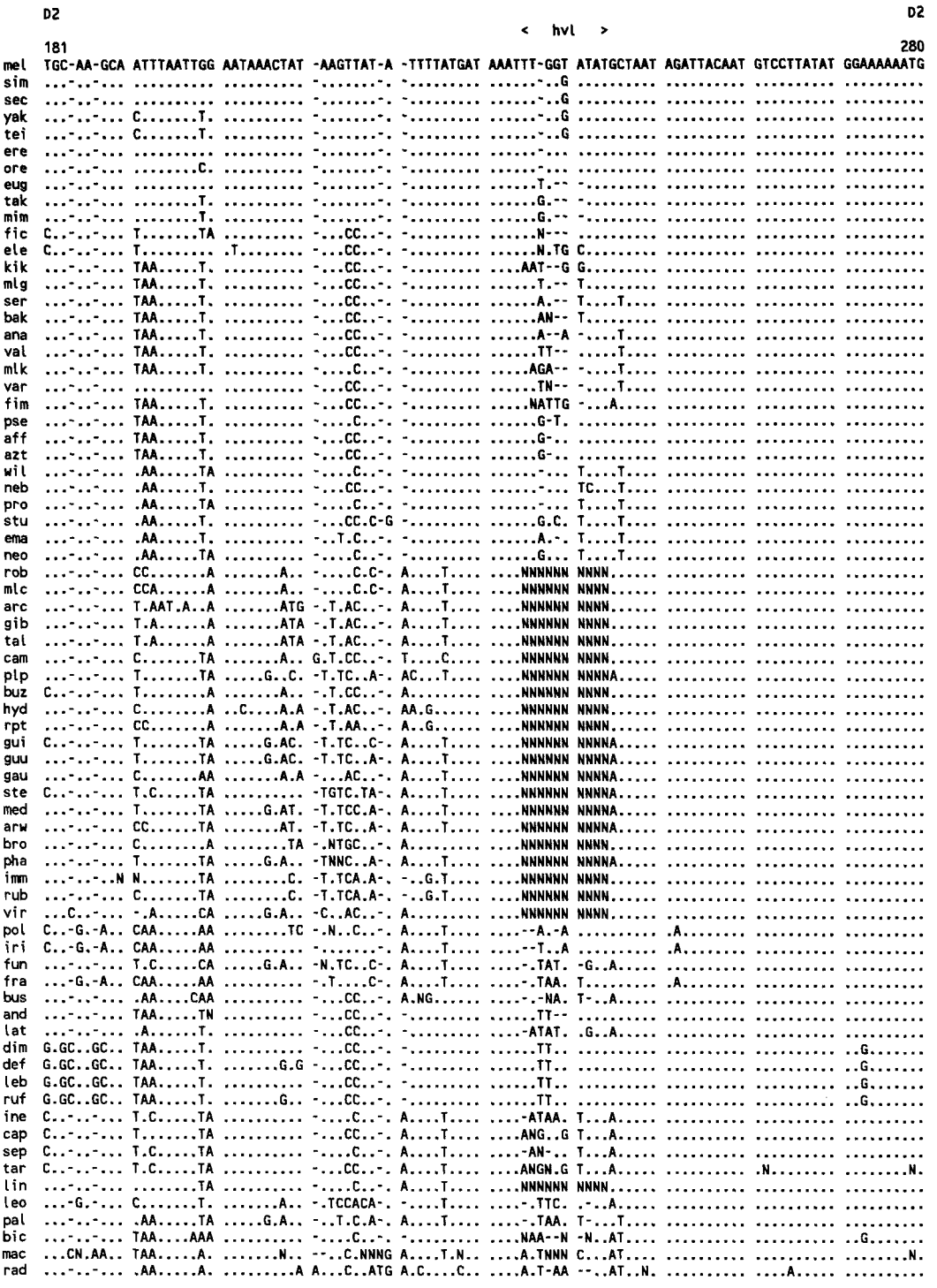

Fig. 1. Continued.

sentially identical to that of the NJ tree represented in Fig. 5. Eight equally parsimonious trees were found with a length of 271 steps for 69 informative sites with a consistency index of 0.52. The major difference between the two methods is the position of the *immigrans* group. According to the parsimony, it is the sister group of the *polychaeta* lineage or the sister group of the *virilis* + *phalerata* lineage.

As long as the subgenus *Drosophila* is considered alone, the species are branched in three lineages, hereafter named *polychaeta, virilis,* and *immigrans-phalerata* respectively. In general, the same species were always clustered in the same lineage, whatever the method of tree construction used. Several sets of subsamples were designed to test the robustness of the three lineages. All the resulting trees calculated by both parsimony and NJ methods gave the same three lineages. The *polychaeta* lineage is

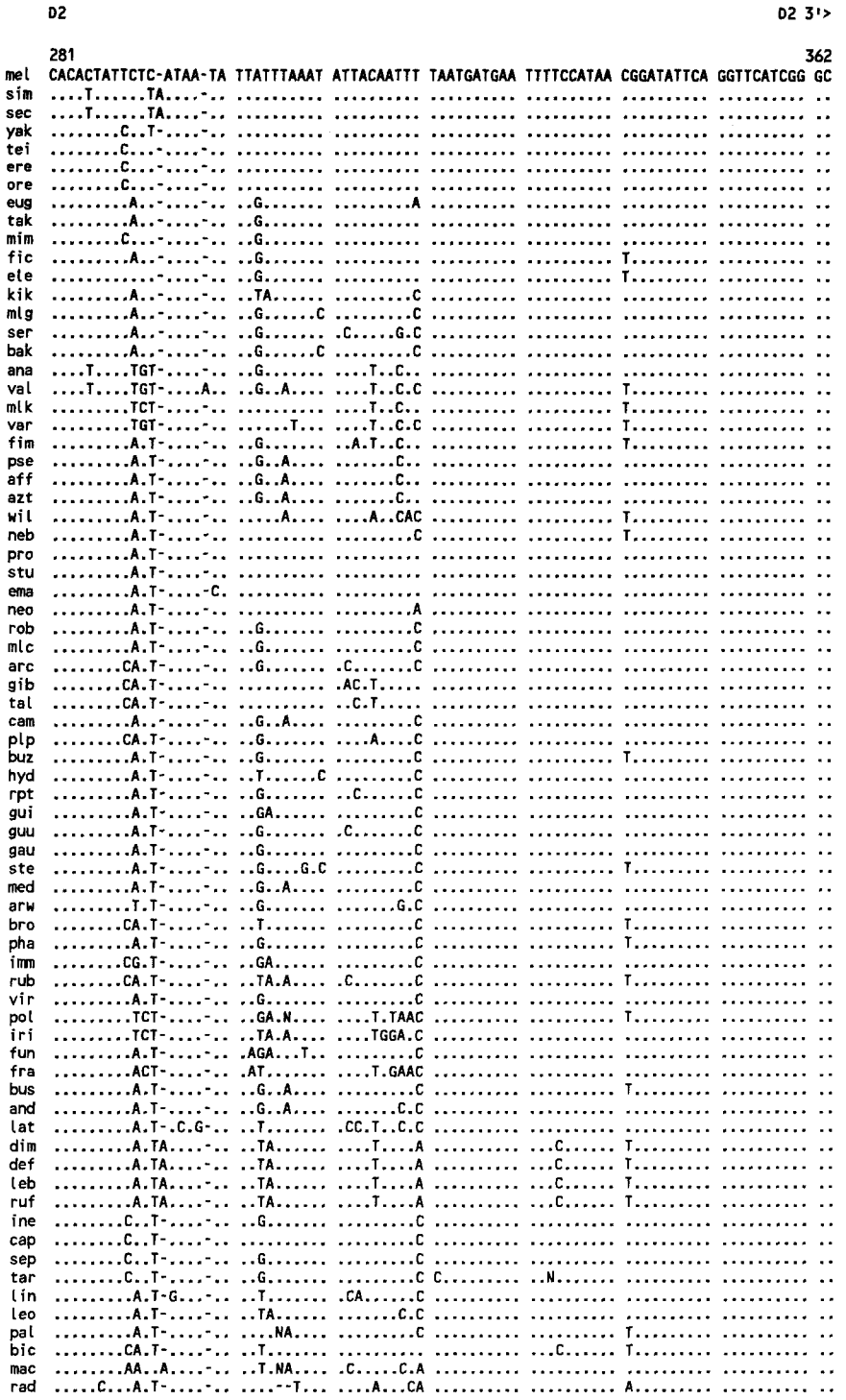

Fig. 1. Continued.

the first to diverge in this trio, this early separation being supported by all methods of analysis.

The *polychaeta* Lineage

The first lineage is constituted, in our sample, by the *polychaeta* species group and the two ungrouped species, *D. fraburu* and *D. iri*. In the bootstrap analysis, *D. polychaeta*, *D. iri*, and *D. fraburu* formed a monophyletic group in 99% of the bootstrap trees. In this lineage, the *polychaeta* species group is more related to *D. iri* than to *D. fraburu*. The close association of these two species was supported by 76% of bootstrap resampling.

The *virilis* Lineage

This lineage includes the *annulimana*, *bromeliae*, *dreyfusi*, *melanica*, *mesophragmatica*, *repleta*, *robusta*, and *virilis* groups. Two sublineages emerged

Table 1. Distance matrix for 39 selected species: the number indicated between each pair of species corresponds to the p distance $\times 10^4$

	tak	kik	bak	ana	var	fim	pse	wil	neb	pro	pol	iri	fra	rob	vir	gib	cam	rep	gau	bro	imm	fun	gua	ste	pal	med	ara	pha	Zin	Zli	Sam	Sca	bus	and	lat	dim	Chy	Leu
(S) melanogaster	150	301	320	432	338	395	376	488	394	524	734	619	791	432	543	570	543	599	525	451	509	583	526	694	638	582	525	546	432	450	677	657	620	536	582	870	712	966
D (S) takahashii		244	244	413	395	357	338	450	413	487	753	600	791	507	562	684	506	599	507	470	472	583	508	657	600	507	469	471	432	413	639	657	602	517	563	870	749	1004
D (S) kikkawai			113	338	413	282	301	432	281	506	659	582	772	488	543	627	506	599	525	489	453	508	451	638	600	469	507	471	450	413	564	600	564	364	525	704	674	947
D (S) bakoue				338	432	282	301	450	338	524	697	619	810	488	524	646	543	618	563	545	528	545	489	600	600	507	544	509	450	488	639	619	564	364	600	796	712	985
D (S) ananassae					244	207	263	469	413	562	697	657	829	582	674	665	674	730	657	696	604	639	620	713	657	582	600	622	544	638	752	619	639	421	638	815	712	985
D (S) varians						301	395	450	394	581	678	657	866	600	693	684	674	674	638	564	604	696	639	750	713	638	582	603	582	600	733	638	639	536	563	852	768	1061
D (S) fima							244	450	394	581	697	657	791	563	618	589	618	655	563	602	604	583	526	619	619	525	544	490	469	544	696	638	508	421	525	741	618	966
D (S) pseudoobscura								413	413	487	697	638	753	544	618	684	599	712	638	639	623	639	583	675	638	544	600	584	488	563	714	582	564	345	638	796	637	909
D (S) willistoni									225	262	658	655	808	562	673	683	654	729	655	600	716	713	675	787	712	581	618	583	562	543	750	506	638	459	599	887	617	1002
D (S) nebulosa										337	639	693	808	468	561	607	561	617	543	488	584	582	544	674	637	506	524	470	487	431	600	468	563	402	468	721	579	945
D (S) prosaltans											769	710	863	579	709	701	690	728	692	712	752	749	712	822	748	654	673	657	598	579	824	617	749	553	692	886	616	1094
D (D) polychaeta											264	510	565	696	630	677	677	584	640	756	698	623	791	772	659	659	679	527	603	735	678	660	672	846	1020	919	1101
D (D) iri												433	544	674	703	693	712	619	677	697	639	564	732	713	638	619	640	488	563	732	675	602	688	768	943	879	1040
D (D) fraburu													584	790	782	752	790	716	774	889	717	736	810	772	772	753	718	659	678	829	810	811	787	940	1113	994	1044
D (D) robusta														431	456	449	431	357	451	490	357	357	563	469	338	394	377	357	375	600	450	583	535	674	887	748	983
D (D) virilis															531	486	486	393	469	639	413	450	655	581	449	562	413	431	468	693	543	600	592	766	980	821	1021
D (D) gibberosa																493	474	399	476	687	591	552	761	627	532	608	496	494	494	741	627	667	637	740	1011	796	1071
D (D) camargoi																	411	281	469	602	563	507	674	599	431	506	395	468	412	524	599	600	534	766	1015	896	1019
D (D) repleta																		318	413	620	600	563	768	693	543	543	470	543	506	655	693	675	687	748	1089	952	1132
D (D) gaucha																			376	565	451	413	638	563	375	413	320	357	338	525	563	526	574	712	1035	822	983
D (D) bromeliae																				509	565	509	658	564	451	470	433	432	413	620	620	622	651	675	981	805	1099
D (D) immigrans																					434	302	509	377	358	358	378	471	509	585	622	679	615	753	1004	846	1122
D (D) funebris																						188	395	376	263	395	283	376	489	545	451	622	594	732	1000	805	1023
D (D) guarani																							395	301	207	301	207	320	432	526	489	584	556	713	944	805	1023
D (D) sternopleuralis																								413	413	469	339	450	619	732	582	677	708	805	1017	748	1078
D (D) pallida																									244	375	282	413	525	694	544	696	631	787	980	804	1153
D (D) mediopictoides																										244	170	338	394	525	375	545	516	655	961	804	1021
D (D) arawakana																											207	413	450	525	525	620	555	618	1017	804	1021
D (D) phalerata																												339	377	471	414	528	537	658	946	732	1006
Z (Z) inermis																													281	544	507	451	497	655	924	636	926
Z (A) lineosus																														507	488	526	497	581	961	785	983
S leonensis																															638	733	632	769	1111	936	928
S pallida																																583	555	787	1035	748	962
D (Do) busckii																																	575	750	1000	712	1023
D (L) andalousiaca																																		669	925	706	944
D (Sc) latifasciaeformis																																			778	749	1040
D (Sc) dimorpha																																				852	1203
C. bicolor																																					1002
L. maculata																																					

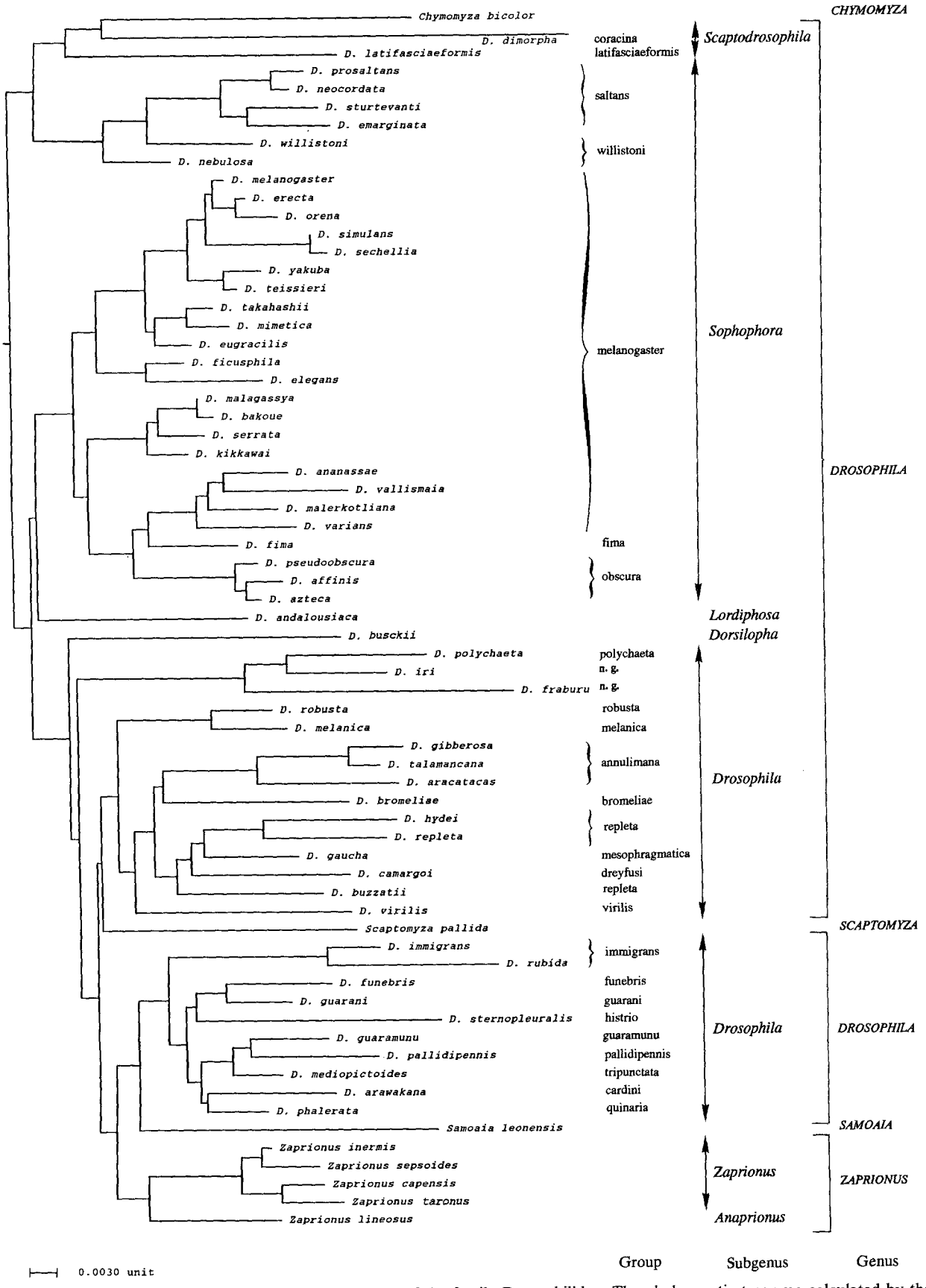

Fig. 2. Phylogenetic relationships among 69 species of the family Drosophilidae. The phylogenetic tree was calculated by the NJ method using the *p* distance. *Delia radicum* and *Leucophenga maculata* were used as outgroup. The classical taxonomical framework is indicated on the right side. *n.g.*: species not grouped.

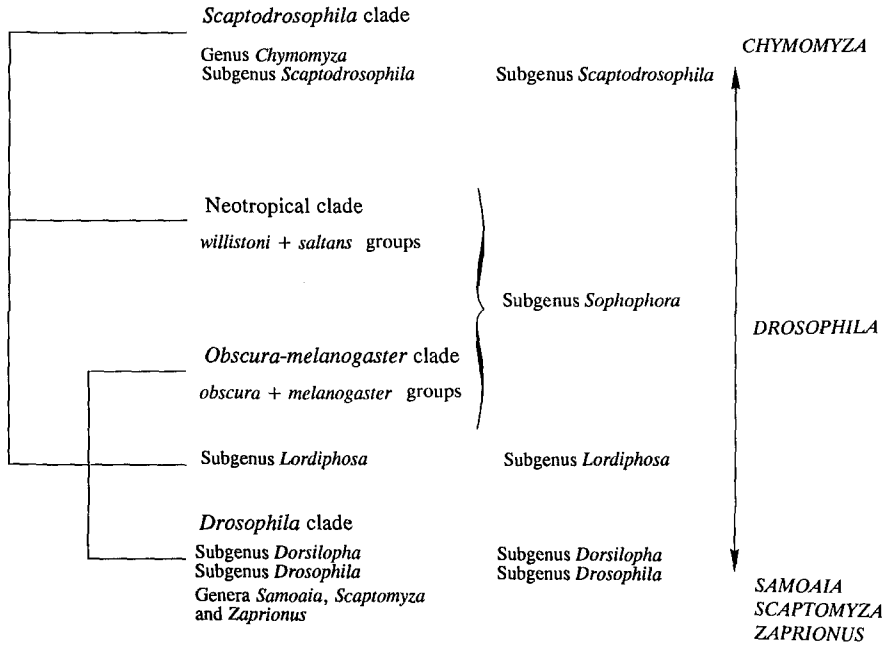

Fig. 3. The consensus tree inferred from the NJ method using the general sample.

in the analyses: the *robusta* and *melanica* groups and the remaining groups. However, *robusta* and *melanica* groups were occasionally branched with the *immigrans-phalerata* lineage. This is reflected by the weak bootstrap score for the *virilis* lineage. This score increased when the *robusta* and *melanica* groups were removed.

Within the second sublineage the *virilis* species group is the first to diverge. *D. camargoi* and *D. gaucha* are branched together very often and constitute the sister group of the *repleta* group (represented here by the three species *D. repleta, D. hydei,* and *D. buzzatii*). The species of the *repleta* group and *D. camargoi* + *D. gaucha* are branched together before being joined by the species of the *annulimana* group. This was shown by parsimony as well as by the NJ tree.

The phylogenetic relationships between *D. bromeliae* and the other members of the lineage are not very clear: it is placed either inside of the sublineage or at the basis of the whole lineage. The monophyly for the three species (belonging to three subgroups) analyzed in the *repleta* group is not always confirmed. *D. repleta* and *D. hydei* are closely related but are not systematically clustered with *D. buzzatii*. The bootstrap tree of the Fig. 5 revealed a relatively low signification for this group (42%). In comparison with the other groups, the monophyly

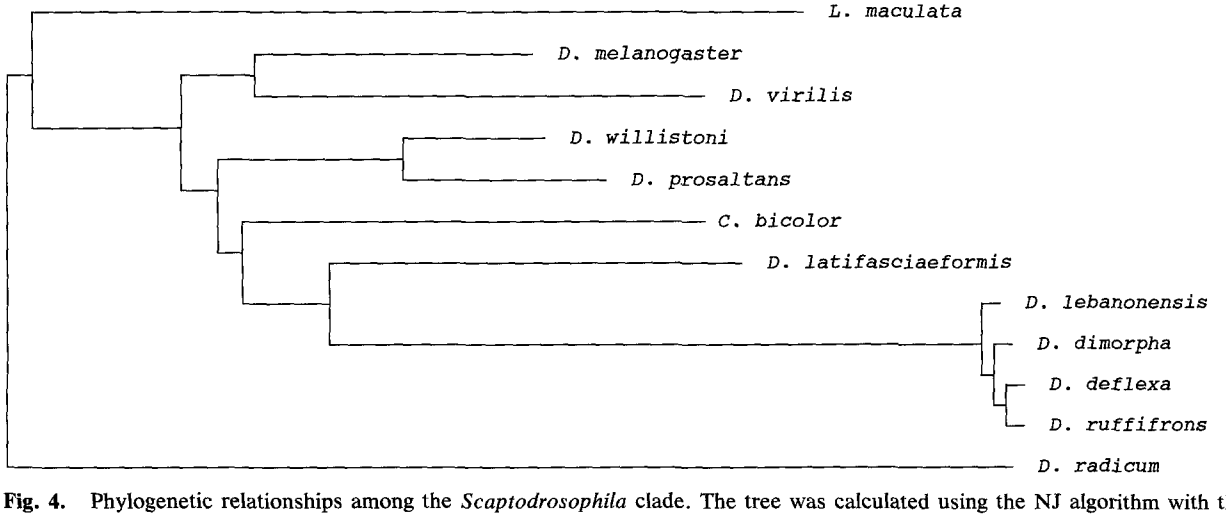

Fig. 4. Phylogenetic relationships among the *Scaptodrosophila* clade. The tree was calculated using the NJ algorithm with the *k* distance. Deletions were weighted as transversions. Note the strong similarities for two groups and the large distance for the *latifasciaeformis* group.

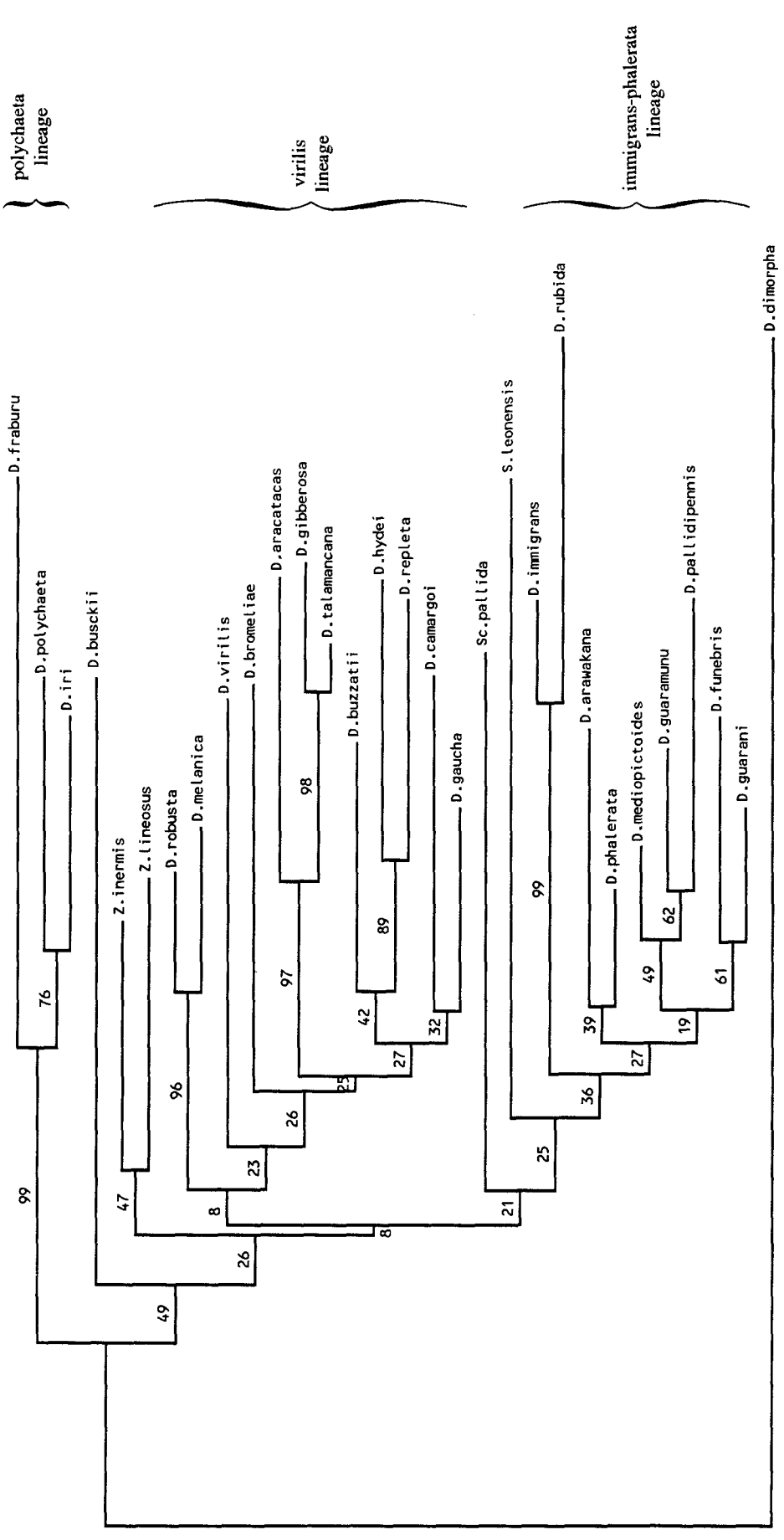

Fig. 5. Phylogenetic relationships among the *Drosophila* clade using *D.* (*Scaptodrosophila*) *dimorpha* as outgroup species. The tree was obtained using the NJ method with the *k* distance. Deletions were weighted as transversions. Numerical values associated with each of the nodes represent the percentage of 1,000 bootstrapped trees.

of the groups *annulimana* and *immigrans* is supported by 97% and 99%, respectively, of the bootstrap replicates.

The *immigrans-phalerata* Lineage

This lineage includes the groups *immigrans, funebris, quinaria, cardini, guaramunu, guarani, histrio, tripunctata,* and *pallidipennis*. Two sublineages were regularly obtained and they are apparent in Fig. 2: the *immigrans* sublineage includes the species studied in the *immigrans* group and the *phalerata* sublineage includes the species of the eight remaining groups. In the *phalerata* sublineage, we can notice three branches: *guarani/funebris, quinaria/cardini,* and *guaramunu/pallidipennis/tripunctata*.

Position of the Genera *Samoaia, Scaptomyza, Zaprionus,* and of the Subgenera *Dorsilopha* and *Lordiphosa*

As shown in Figs. 2 and 5, the species representative of the genera *Zaprionus, Samoaia,* and *Scaptomyza* are not only internal branches of the genus *Drosophila* but are also included in the subgenus *Drosophila*. However, the phylogenetic relationships of the three genera with the different lineages of the subgenus *Drosophila* are not resolved. Nevertheless, it should be noted that they generally emerged after the separation of the *polychaeta* lineage and that *Samoaia leonensis* is very closely related to the *phalerata* lineage. Concerning the genus *Zaprionus,* five species have been sequenced: four belonging to the subgenus *Zaprionus* are clustered together and only one representing the current subgenus *Anaprionus, Z. (A.) lineosus,* previously named *Drosophila (Drosophila) lineosa* and ranged within the *immigrans* group.

The position of *D. (Dorsilopha) busckii* is far from clear: this species was sometimes placed among the *Drosophila* clade as the most ancient lineage or just after the node of the *polychaeta* lineage. Similarly, the phylogenetic position of *D. (Lordiphosa) andalousiaca* is not stable. In the various analyses, this species was found close to the subgenus *Sophophora* (with reduced samples), close to the *Drosophila* clade, or as a sister group of the *obscura-melanogaster* clade as in Fig. 2.

Discussion

The 63 *Drosophila* species sequenced for this study represent the most comprehensive sample used in biochemical studies but only represent 5% of the species of this genus. Only the subgenera *Drosophila, Scaptodrosophila,* and *Sophophora* have been examined with a reasonable set of species (respectively, 25, 5, and 31 species).

Tree Construction Strategy

Topology of phylogenetic trees depends on several parameters—namely, the general method and the particular algorithm, the set of species used representing the different taxa (the outgroups often being of the greatest importance), as well as the pattern of nucleotide substitution and the length of the sequences. The robustness of the phylogeny depends on the level of homoplasy and information redundancy, but also on the number of species analyzed and their pattern of branching: a tree with regularly spaced nodes with few species is generally easier to confirm than dense and bushy trees.

We have tested the robustness of the phylogenetic reconstructions, based on 553 nucleotides of the 28S rRNA gene in 72 drosophilid species, by subsampling the sites with the bootstrap test, by eliminating the hypervariable sites, by subsampling the species representative of the outgroups or the internal taxa, and by analyzing separately particular clades or lineages. In most of the cases, a variety of parsimony and distance methods was also applied.

For the general tree, the choice of an appropriate outgroup has been difficult. The apparently simplest solution—i.e., a choice guided by the taxonomical position of one or several drosophilines belonging to genera related to *Drosophila*—is not necessarily correct. Most of the genera, although occasionally used as outgroups (for instance, *Chymomyza* by Beverley and Wilson 1982) have been shown in the present study, as well as in some of the previous ones (Throckmorton 1975; Grimaldi 1990), to be in fact internal to the genus *Drosophila* itself. Consequently, in spite of its remoteness, we have used *Leucophenga maculata,* a member of Steganinae, the sister subfamily of Drosophilinae in Drosophilidae, as an outgroup. The external position of *L. maculata* was confirmed by the use of *Delia radicum* (Antomyiidae) or *Calliphora vomitaria* (Calliphoridae) (data not shown) and of species of more distant families (data not shown)—*Ceratitis capitata* (Tephritidae) and *Glossina tachinoides* (Glossinidae). For the study of the different clades and lineages subsampled among the species sequenced, we have used as outgroups various species selected after inspection of the general tree.

In the D1 and D2 sequences, the pattern of substitution is not random in several ways (Rousset et al. 1991): Some sites are strictly invariant, while others are hypervariable (particularly in the loops); there is a high proportion of transitions in the helices of the secondary structures (about half of the nucleotides of the sequences are paired) in relation with the dominant pattern of compensatory substitutions: G-C ↔ G.U ↔ A-U; in addition, the sub-

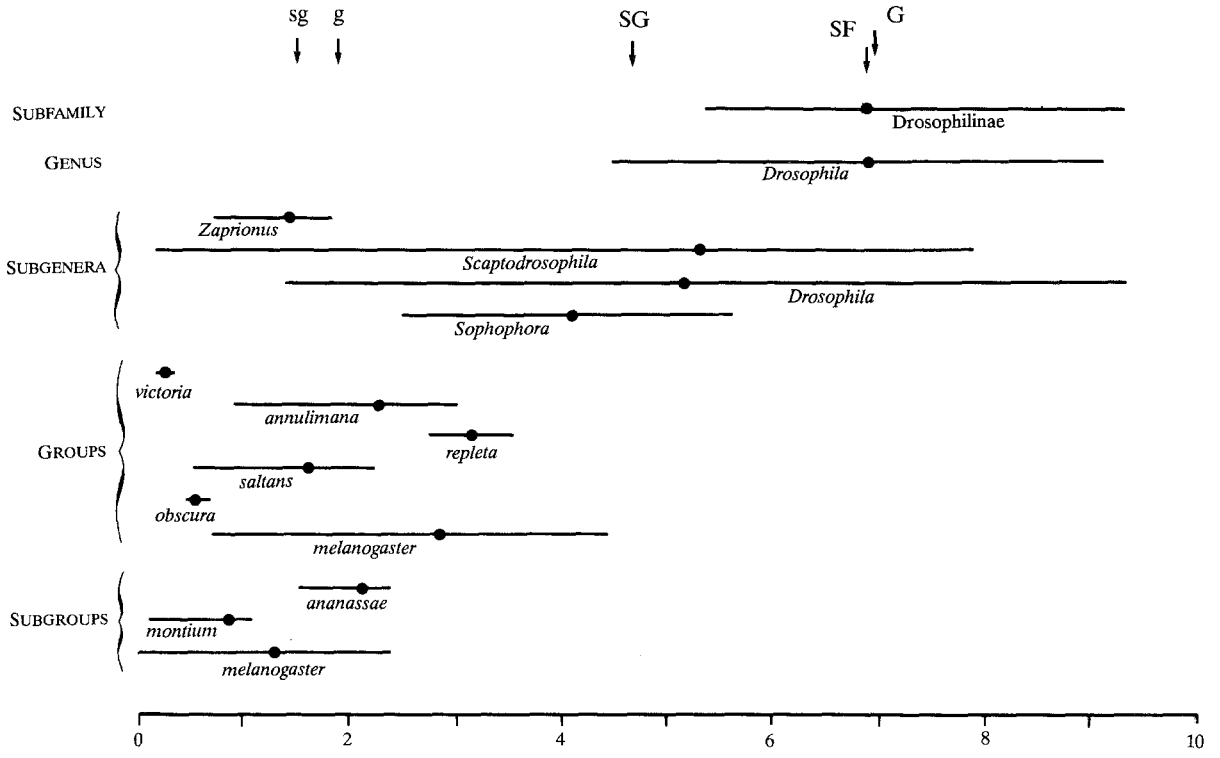

Fig. 6. Variability of the nucleotide distance for different taxa in the subfamily Drosophilinae. The range (*bar*) and the average (*circle*) are indicated for each case. In order to weight the distances for groups overrepresented by numerous species, the distances between taxa are those averaged for their immediate subordinates. Abscissa: nucleotide divergence in percent; ordinates: taxa. Distance between species within *arrows* indicate the average values. Distances based on a single value are not given.

stitutions in the stems are not independent. Consequently, in spite of the reasonable length of the sequences obtained for each of them (about 560 bases) and of the large number of variable sites (204 sites), the homoplasy obscured the significance of some of the nodes. However, a lot of new and interesting features emerged from the phylogenetic trees of the 72 Drosophilidae species sequenced, particularly for the higher taxa, i.e., the subgenera of *Drosophila* and the genera related to *Drosophila*.

Nucleotide Distances

The average nucleotide distance between taxa of various rank and the range of their variation were deduced from the complete distance matrix (not shown). (See Fig. 6.) The distances were first calculated between species of the same subgroup, then between subgroups (average of specific distances), then between groups as the average distances between their subgroups if any, and so on. The survey of the distances shows that their mean increases with the rank of the taxa. However, there is a strong heterogeneity between the distances of taxa of the same rank and some inconsistencies. They can have several origins.

One potential origin is the variability of the rate of evolution from one lineage to another. This is illustrated by Fig. 7, and it is also apparent in Fig. 2 that, even for related species, the patristic length of the branches is far from uniform, the difference reaching a factor of four for *D. nebulosa* and *D. emarginata*. Few species (e.g., *D. dimorpha, D. fraburu,* and *D. rubida*) have evolved very rapidly, whereas most species form the main bulk of the distribution. For the total, the average is 16.8 and the variance 20.8, this last figure being only 1.2 times that of the mean. Consequently the heterogeneity of evolutionary rates is less important than that of many genes (Wilson et al. 1977).

For most of the species, we have no additional information, but a previous study of the *obscura* group (with a larger number of species than here), and comparison to the *melanogaster* subgroup, showed that the ribosomal RNA does not reflect the general rate of evolution. The ratio of evolutionary rates in *obsura/melanogaster* depends on the compartment considered: it was about 0.5 for rRNA, 1 for allozymes (Nei distance), and 2 for mtDNA (Ruttkay et al. 1992). With such heterogeneities between compartments, we can hardly try to correlate evolutionary rates with ecogeographical characteristics of the groups. The heterogeneity of the subgenera *Drosophila* and *Scaptodrosophila* is also striking.

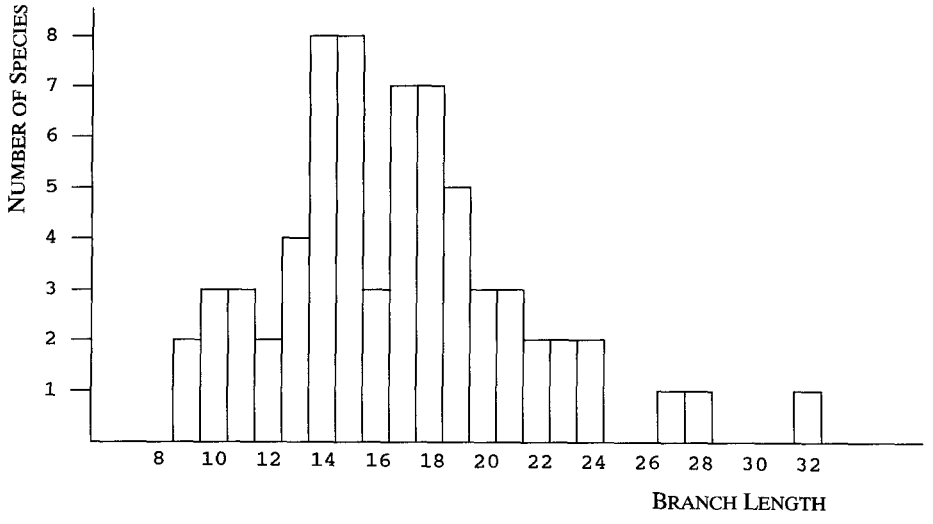

Fig. 7. Distribution of the frequencies of the branch lengths grouped in classes showing the number of species corresponding to each class. The patristic distances were deduced from the general tree of the Fig. 1.

Another source of discrepancy between distance and taxonomical rank is the existence of a large number of paraphyletic groups. This is important mainly for higher taxonomical levels. Clearly, the variability within the genus *Zaprionus* is very small (the distance value is 3.66% for the most distant species) compared to *Drosophila*. This lower variability, similar to that of a group of species, is less surprising if, following our results, the genus *Zaprionus* is considered as an internal branch of a subgenus of *Drosophila*.

Phylogenetic Relationships at the Generic and Subgeneric Level

The general tree, shown in Fig. 2, encompassing the subgenera *Scaptodrosophila, Sophophora, Drosophila, Lordiphosa,* and *Dorsilopha* of the genus *Drosophila* and the genera *Chymomyza, Scaptomyza, Zaprionus,* and *Samoaia* shows some differences in comparison with those derived from morphological analyses (Throckmorton 1975; Okada 1989; Grimaldi 1990) or molecular data (Beverley and Wilson 1982; Zweibel et al. 1982; Caccone et al. 1992; DeSalle 1992).

According to Throckmorton (1975), the four genera listed above are branched inside of the genus *Drosophila*. *Chymomyza* is a member of his Sophophoran radiation while the other three genera belong to the *Drosophila* radiation (which itself emerges from the Sophophoran radiation). According to Okada (1989), the genus *Drosophila* should be considered as a monophyletic genus and all the other genera considered here are placed outside of it. The cladistic analysis derived from morphological data (Grimaldi 1990) places these genera outside of the subgenera *Sophophora* and *Drosophila* but after the emergence of the subgenus *Scaptodrosophila*. Because he considers this subgenus as a separate genus, Grimaldi (1990) suggests therefore that *Chymomyza, Zaprionus, Samoaia,* and *Scaptomyza* have branched out of the genus *Drosophila*. This phylogenetic position of *Chymomyza* is also found by DeSalle (1992). Our results are only in partial agreement with those of DeSalle (1992) and Grimaldi (1990) because, in our study *Chymomyza* is not branched between *Sophophora* + *Drosophila* and *Scaptodrosophila* but is associated with this last subgenus. *Drosophila lineosa,* previously assigned to the subgenus *Drosophila,* is currently classified in the genus *Zaprionus,* subgenus *Anaprionus: Z. (A) lineosus*. The position of the genus *Zaprionus* in the clade is in agreement with this classification.

Concerning the genus *Scaptomyza,* the conclusions of the different morphological analyses (Throckmorton 1975; Okada 1989; Grimaldi 1990) are again not similar. This genus is integrated into the subgenus *Drosophila* by the first author and considered as a separate genus by the two others although their phylogenetic conclusions are different: *Scaptomyza* is branched outside of the subgenus *Drosophila* and *Sophophora* by Grimaldi and branched outside of the three main subgenera of *Drosophila* by Okada. The rRNA tree, like other molecular studies (Beverley and Wilson 1982; DeSalle 1992) and the conclusion of Throckmorton (1975), supports *Scaptomyza* being internal to the subgenus *Drosophila*.

The genus *Zaprionus* is considered as a member of the *Drosophila* radiation by Throckmorton (1975) but branched outside of the subgenus *Drosophila* and *Sophophora* by Grimaldi (1990). It has rarely been included in the molecular approaches of phylogeny: recently, as shown in Fig. 8, DeSalle (1992) obtained results comparable to Grimaldi. However,

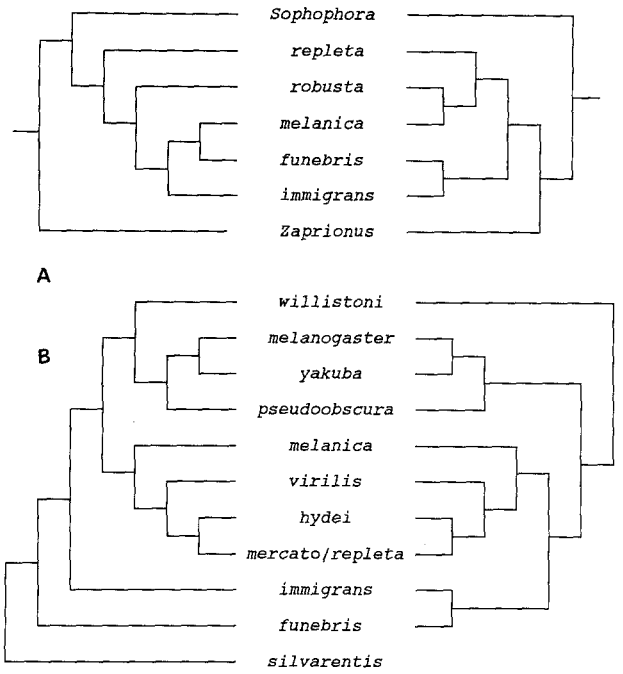

Fig. 8. **A** Comparison of the rRNA results (on the right side) with those of DeSalle (1992) (on the left side). Both trees rooted by *Chymomyza* were deduced from the parsimony method. The species used to represent the various taxa are not the same in the two analyses. **B** Comparison of the rRNA results (on the right side) to those of Caccone et al. (1992) (on the left side). Both trees were deduced from the distance matrix using the NJ method. The topology we presented is inferred from the general NJ tree. We have used *D. repleta* instead of *D. mercatorum* and *D. silvarentis* is not available in our sample.

according to Maruyama and Hartl (1991), *Zaprionus* is branched within the genus *Drosophila*. This is also the result of our study, in spite of its instability within the *Drosophila* clade.

As long as the genus *Drosophila* is considered alone, the relationships between the three main subgenera *Scaptodrosophila, Sophophora,* and *Drosophila* are in general agreement with the other studies (earlier emergence of *Scaptodrosophila*), the principal difference being the existence, for the rRNA phylogenies, of two separate clades in the subgenus *Sophophora,* i.e., the Neotropical and the *obscura-melanogaster* clades, the subgenus *Drosophila* being branched with the last one (Pélandakis et al. 1991). This is the only controversial point, the subgenus *Drosophila* being the sister group of *Sophophora* in the other phylogenies.

The phylogenetic position of the two other subgenera we have analyzed—namely, *Dorsilopha* and *Lordiphosa*—is far from clear. Lastovka and Macà (1978) revised the taxonomy of the subgenus *Lordiphosa* and considered it as closely related to the subgenus *Sophophora*. This is the position of *D. (Lordiphosa) andalousiaca* as shown in the general tree, but this position is not very stable. Other authors place the subgenus *Lordiphosa* close to the genus *Scaptomyza* (Hackman 1982; Grimaldi 1990). The position of *D. (Dorsilopha) busckii,* branching within the subgenus *Drosophila* in our results, is in agreement with that of Throckmorton (1975).

Phylogeny Within the Subgenera

According to Bock (1978), the subgenus *Scaptodrosophila* established and previously termed *Pholadoris* by Sturtevant (1942), is "probably the least well understood of the major *Drosophila* subgenera." Among the 224 species included in this subgenus, only 70 species have been ranged in 9 separate groups (Bock 1978; Tsacas et al. 1988). In the rRNA analysis, the *coracina* and *victoria* groups appeared very closely related: the range of nucleotide distance between their species (0.19% to 0.37%) evokes more species of the same subgroup than species of different groups. In return, the distance between those species and *D. latifasciaeformis* is very high, averaging almost 8%.

Although the *Sophophora* tree is not very robust, as judged by the bootstrap scores, most of the taxa designed by traditional systematics appear as monophyletic groups in the rRNA phylogenetic tree (Pélandakis et al. 1991). The noticeable results are (1) the early separation of the Neotropical clade; (2) the *ananassae* subgroup is not branched with the other subgroups of the *melanogaster* group, but with the *obscura* group; (3) The position of the *fima* group with the *ananassae* subgroup was unpredictable since its phylogenetic relationsips with the species group of *Sophophora* are obscure.

According to Throckmorton (1975), the *Drosophila* radiation is divided into two subradiations, *virilis-repleta* and *immigrans-tripunctata;* these correspond to the *virilis* and *immigrans-phalerata* lineages of Fig. 5. The only major difference is the early emergence of *D. polychaeta, D. fraburu,* and *D. iri* as an independent monophyletic lineage. Within the *polychaeta* lineage, *D. iri* is closer to *D. polychaeta* than to *D. fraburu*. However, few studies have been carried out on them. It is generally admitted that *D. iri* and *D. fraburu* are closely related (Burla 1954; Vouidibio 1977). The phylogenetic position of *D. polychaeta, D. iri,* and *D. fraburu* revealed by their rRNA study is not in agreement with the opinion of Throckmorton (1962), for whom *D. polychaeta* is a member of the *virilis-repleta* radiation. This result was rather surprising for us but it has received strong support from the analysis of Beppu who, on the basis of morphological studies, deduced the monophyly of this group (personal communication to L. Tsacas).

With the exception of these three species, the two lineages *virilis* and *immigrans-phalerata* are

also supported by other data (Beverley and Wilson 1982; Spicer 1988; Grimaldi 1990). However, several differences appear when the topologies within the main lineages of *Drosophila* are considered. For the *virilis* lineage, in comparison with other studies, the major difference is the position of the *robusta* and *melanica* groups. According to our results they are very closely related and are sister groups of the rest of the groups belonging to the *virilis* lineage. This phylogenetic position is slightly supported by the bootstrap procedure but appeared recurrently in the variant trees. According to the other molecular phylogenetic trees (Beverley and Wilson 1982; Spicer 1988; DeSalle 1992) the relationships of *robusta* and/or *melanica* groups are controversial. The close association of the *robusta* and *melanica* groups we have found is accepted by some authors (Stalker 1972; Levitan 1986) but not by others (Throckmorton 1982; Spicer 1988; DeSalle 1992). The relationship of the *repleta* group within the *virilis* lineage is also supported by Throckmorton (1982). The *repleta* group is closely related to the *dreyfusi* and *mesophragmatica* groups. The fact that this group is the most derived taxon in the subgenus *Drosophila* was also supported by the results of Beverley and Wilson (1982), Spicer (1988), Grimaldi (1990), and Caccone et al. (1992). On the contrary, DeSalle (1992) found that the *repleta* group is the first to diverge among the subgenus *Drosophila* (Fig. 8). The monophyly of the *repleta* group is not demonstrated with respect to the morphological studies (Throckmorton 1962).

The *immigrans-phalerata* lineage defined by the rRNA phylogeny corresponds well to the *immigrans-Hirtodrosophila* radiation of Throckmorton (1975) and the rRNA tree reveals two sublineages. The *immigrans* group is alone in the first one and the second, *phalerata*, is made up of the *cardini, funebris, guaramunu, guarani, histrio, pallidipennis, quinaria* (including *D. phalerata*), and *tripunctata* groups. Within the *phalerata* lineage, the topology is relatively stable. The main discrepancy with the other studies concerns the position of the *funebris* group. We found it closely related to the *guarani* group, and this pair is clearly associated with the *phalerata* lineage. In the literature the position of this group is controversial. Caccone et al. (1992) and DeSalle (1992) give a branching pattern very different for this species, as shown in Fig. 8. According to Throckmorton (1975), it is considered as an intermediate group between its two main radiations. Grimaldi's topology (1990) shows a branching order where this group is the sister group of the other species of the subgenus *Drosophila*. Using molecular data, Spicer (1988) agreed with Throckmorton's conclusion.

Direct RNA sequencing is an efficient method of studying the phylogeny of the Drosophilidae for different reasons. First, we can obtain a great number of sequences of species. This is necessary with regard to the diversity of this family. Second, the analysis of different divergent domains allows us to study the phylogeny of species of various taxa. The rRNA phylogenetic result is surprising with respect to other studies. In particular the monophyly of the subgenus *Sophophora* is not demonstrated in our results. Concerning the subgenus *Drosophila,* the branching pattern is more in agreement with the classical one. The major difference is the position of *D. polychaeta, D. iri,* and *D. fraburu,* which are placed as the most ancient lineage with respect to the other members of the *Drosophila* clade.

This rRNA phylogenetic tree is not definitive. Several nodes are not resolved. The branching pattern of the Neotropical, *obscura-melanogaster, Scaptodrosophila,* and *Drosophila* clades is not stable. In order to resolve the deep nodes, the extension of the sequences to other variable domains will be necessary.

Acknowledgments. We thank D. Vautrin for invaluable technical assistance. We are very grateful to J. Guadet, M. Dutertre, J. Julien, and Y. Brygoo for their help in the beginning of this work. We thank A. Adoutte and H. Philippe for providing the MUST program, and L. Tsacas, H. Ruttkay, F. Rousset, and M. Cobb for comments on the manuscript. We also thank E. Brunel, who provided *Delia radicum;* M. Wegnez, who provided *Myatropa florea;* and J. R. David, who provided *Leucophenga maculata* and several *Drosophila* strains. EMBL database accession numbers for ribosomal RNA sequences D1 and D2 of *Drosophila* are X71158 to X71303.

References

Ashburner M (1989) The family Drosophilidae. In: *Drosophila* a laboratory handbook. Cold Spring Harbor Laboratory Press, New York, pp 1075–1160

Beverley SM, Wilson AC (1982) Molecular evolution in *Drosophila* and the higher Diptera. I. Microcomplement fixation studies of a larval hemolymph protein. J Mol Evol 18:251–264

Beverley SM, Wilson AC (1984) Molecular evolution in *Drosophila* and the higher Diptera. II. A time scale for fly evolution. J Mol Evol 21:1–13

Bock IR (1978) The subgenus *Scaptodrosophila* (Diptera: Drosophilidae). Syst Entomol 3:91–102

Burla H (1954) Zur Kenntnis der Drosophiliden der Elfenbeinküste (Französisch West-Afrika). Rev Suisse Zool 61:1–218

Caccone A, Gleason JM, Powell JR (1992) Complementary DNA-DNA hybridization in *Drosophila*. J Mol Evol 34:130–140

DeSalle R, Grimaldi DA (1991) Morphological and molecular systematics of the Drosophilidae. Annu Rev Ecol Syst 22: 447–475

DeSalle R (1992) The phylogenetic relationships of flies in the family Drosophilidae deduced from mtDNA sequences. Mol Phyl Evol 1:31–40

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Grimaldi DA (1990) A phylogenetic revised classification of genera in the Drosophilidae (Diptera). Bull Am Mus Nat Hist 197:1–139

Hackman W (1982) The relation between the genera *Scaptomyza* and *Drosophila* (Diptera, Drosophilidae). Ann Entomol Fenn 49:97–104

Hassouna N, Michot B, Bachellerie JP (1984) The complete nucleotide sequence of mouse 28S rRNA gene. Implications for the process of size increase of the large subunit rRNA in higher eukaryotes. Nucleic Acids Res 12:3563–3583

Higgins DG, Sharp PM (1988) CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene 73:237–244

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Lastovka P, Macà J (1978) European species of the *Drosophila* subgenus *Lordiphosa* (Diptera, Drosophilidae). Acta Entomol Bohemoslov 75:404–420

Levitan M (1986) The *robusta* and *melanica* groups. In: Ashburner M, Thompson JN, Carson HL (eds) The genetics and biology of *Drosophila* vol 3b. Academic Press, London, pp 141–192

Maccecchini ML, Rudin Y, Blobel G, Schatz G (1979) Import of proteins into mitochondria: precursor forms of extramitochondrially made F1-ATPase subunits in yeast. Proc Natl Acad Sci USA 76:343–347

MacIntyre RJ, Collier GE (1986) Protein evolution in the genus *Drosophila*. In: Ashburner M, Thompson JN, Carson HL (eds) The genetics and biology of *Drosophila,* vol 3e. Academic Press, London, pp 39–146

Maruyama K, Hartl DL (1991) Evidence for interspecific transfer of the transposable element mariner between *Drosophila* and *Zaprionus*. J Mol Evol 33:514–524

Michot B, Bachellerie JP (1987) Comparisons of large subunit rRNAs reveal some eukaryote-specific elements of secondary structure. Biochimie 69:11–23

Okada T (1989) A proposal of establishing tribes for the family Drosophilidae with keys to tribes and genera (Diptera). Zool Sci 6:391–399

Pélandakis M, Higgins DG, Solignac M (1991) Molecular phylogeny of the subgenus *Sophophora* of *Drosophila* derived from the large subunit of ribosomal RNA sequences. Genetica 84:87–94

Qu LH, Michot B, Bachellerie JP (1983) Improved methods for structure probing in large RNAs: a rapid "heterologous" sequencing approach is coupled to the direct mapping of nuclease accessible sites. Application to the 5′ terminal domain of eukaryotic 28S rRNA. Nucleic Acids Res 11:5903–5920

Rousset F, Pélandakis M, Solignac M (1991) Evolution of compensatory substitutions through G.U intermediate state in *Drosophila* rRNA. Proc Natl Acad Sci USA 88:10032–10036

Ruttkay H, Solignac M, Sperlich D (1992) Nuclear and mitochondrial ribosomal RNA variability in the *obscura* group of *Drosophila*. Genetica 85:143–179

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

Spicer GS (1988) Molecular evolution among some *Drosophila* species groups as indicated by two-dimensional electrophoresis. J Mol Evol 27:250–260

Stalker HD (1972) Intergroup phylogenies in *Drosophila* as determined by comparisons of salivary banding patterns. Genetics 70:457–474

Sturtevant AH (1942) The classification of the genus *Drosophila* with descriptions of nine new species. Univ Tex Publ 4213: 5–51

Sullivan DT, Atkinson PW, Starmer WT (1990) Molecular evolution of the alcohol dehydrogenase genes in the genus *Drosophila*. In: Hecht MK, Wallace B, MacIntyre RJ (eds) Evolutionary biology, vol 24. Plenum, New York, pp 107–147

Swofford DL (1990) Phylogenetic analysis using parsimony (version 3.0). Illinois Natural History Survey, Champaign

Tautz D, Hancock JM, Webb DA, Tautz C, Dover GA (1988) Complete sequences of the rRNA genes of *Drosophila melanogaster*. Mol Biol Evol 5:366–376

Throckmorton LH (1962) The problem of phylogeny in the genus *Drosophila*. Univ Tex Publ 6205:207–343

Throckmorton LH (1975) The phylogeny, ecology and geography of *Drosophila*. In: King RC (ed) Handbook of genetics, vol 3. Invertebrates of genetic interest. Plenum Press, New York, pp 421–469

Throckmorton LH (1982) Pathways of evolution in the genus *Drosophila* and the founding of the *repleta* group. In: Barker JFS, Starmer WT (eds) Ecological genetics and evolution. Academic Press, Australia, pp 33–47

Tsacas L, Chassagnard MT, David JR (1988) Un nouveau groupe d'espèces afrotropicales anthophiles dans le sous-genre *Scaptodrosophila* du genre *Drosophila* (Diptera: Drosophilidae). Ann Soc Ent Fr (N S) 24:181–202

Vouidibio J (1977) Biologie évolutive et écophysiologie comparée de deux espèces de drosophiles africaines: *Drosophila iri* et *Drosophila fraburu* (Diptères—Drosophilidae). Thèse d'université Lyon, 141 p

Wilson AC, Carlson SS, White TJ (1977) Biochemical evolution. Annu Rev Biochem 46:573–639

Zwiebel LJ, Cohn VH, Wright DR, Moore GP (1982) Evolution of single-copy DNA and the ADH gene in seven drosophilids. J Mol Evol 19:62–71