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PHYLOGENETIC RELATIONSHIPS OF FLIES IN THE FAMILY DROSOPHILIDAE INFERRED BY COMBINED ANALYSIS OF MOLECULAR AND MORPHOLOGICAL DATA SETS

by

Patrick M. O'Grady II

A Dissertation Submitted to the Faculty of the

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For the Degree of

DOCTOR OF PHILOSOPHY

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entitled Phylogenetic relationships of Flies in Family Drosophilidae Inferred from Combined Analysis of Morphological and Molecular Characters

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Margaret G. Kidwell	5/21/98 Date
William B. Heed	5-15-98 Date
Danny Brower	5/15/98 Date
David Maddison	15 May 1998 Date
J. Bruce Walsh	5/15/98 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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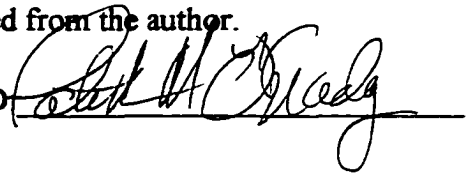
SIGNED 

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ABSTRACT

The phylogenetic relationships of flies within the family Drosophilidae are studied in four clades placed at three different taxonomic levels. The goal of examining phylogenetic relationships of nested clades within Drosophilidae is to study patterns of divergence at several points, giving a more informative view of evolution in this family of flies. Two studies presented here examine the phylogenetic relationships within the *Drosophila saltans* and *Drosophila obscura* species groups. Another examines the relationships among several species groups within the subgenus *Sophophora*. The final study examines the phylogenetic relationships among genera within the family Drosophilidae.

The *saltans* species group consists of approximately 20 species, all distributed in the Neotropical region. This species group has been divided into five species subgroups, based on morphological characters. However, the relationships among the subgroups have either never been tested using an explicit phylogenetic methodology or are incongruent with one another. In this study I examine the phylogenetic relationships within and among the five subgroups in the *Drosophila saltans* species group using nucleotide sequences from two mitochondrial loci, cytochrome oxidase I and II, and two

nuclear loci, alcohol dehydrogenase and the internal transcribed spacer of the nuclear ribosomal DNA repeats.

The molecular data gathered for members of the *Drosophila saltans* species group are congruent with the conclusions of previous taxonomic studies. All five species subgroups are monophyletic with respect to one another. Relationships among the subgroups indicate that the *neocordata* and *elliptica* subgroups are basal in the saltans species group. The *saltans* and *parasaltans* subgroups are derived sister taxa and the *sturtevanti* subgroup occupies an intermediate position within the *saltans* species group.

The *obscura* species group contains approximately 50 species and has a mostly Holarctic distribution, with some species being found in Old and New World tropical regions. The *obscura* species group has been studied extensively over the past 40 years and, while progress has been made in the understanding of the phylogenetic relationships among species in the *obscura* group, many studies are incongruent with one another. The *obscura* group study examines the phylogenetic relationships within and among the *obscura*, *subobscura*, *microlabis*, *pseudoobscura* and *affinis* subgroups using nucleotide sequences from six mitochondrial (*16S*, cytochrome b, cytochrome oxidase I and II, NADH dehydrogenase I and V) and five nuclear (*5S*, *28S*, alcohol dehydrogenase, glycerol-3-phosphate dehydrogenase, superoxide dismutase) loci.

The results of the *Drosophila obscura* analyses indicate that all five of the subgroups are monophyletic with respect to one another. Furthermore, the New and Old World *obscura* species groups are monophyletic, a result which has been suggested by some data in the past. This study also suggests, albeit weakly, that the Afrotropical *microlabis* subgroup may be the sister taxon of the *subobscura* subgroup.

Another study looks at relationships among five species groups placed within the subgenus *Sophophora*. There have been several treatments, both morphological and molecular, dealing with the evolutionary relationships of *Sophophora*. The majority indicate that all subgroups within *Sophophora*, as well as *Sophophora* itself, are monophyletic. However, recent studies, which used the *28S* locus to examine relationships among the *melanogaster*, *obscura*, *saltans*, *willistoni* and *fima* species groups, are incongruent with this notion. These data indicate that, in addition to the *melanogaster* species group being paraphyletic with respect to the *obscura* and *fima* subgroups, the entire subgenus was paraphyletic with respect to the subgenus *Drosophila*. In this study I use using nucleotide sequences from the mitochondrial cytochrome oxidase II, nuclear alcohol dehydrogenase and nuclear *28S* loci to determine the phylogenetic relationships within and among the *melanogaster*, *obscura*, *saltans*, *willistoni*, and *fima* species groups.

The results of this study indicate that *Sophophora* is monophyletic with respect to the subgenus *Drosophila*. Within *Sophophora*, the *obscura* and *saltans* species groups are highly supported as monophyletic. The *willistoni* and *melanogaster* species groups, however, may not be monophyletic. This is possibly due to rapid speciation at the base of these two species groups.

The final study uses extensive field collections, combined with specimens obtained from the National *Drosophila* Species Stock Center, to examine the higher-level relationships among sixty major clades within the family Drosophilidae. Although previous studies have indicated that phylogenetic relationships based on molecular and morphological characters are incongruent with one another, expanded taxon sampling and reanalysis indicates that these characters are not in conflict. Determining the sister clade of the Hawaiian Drosophilidae, as well as relationships within this incredibly diverse clade, is one focus of this study.

The relationships inferred through combined analysis of all available molecular and morphological data indicate that the genus *Drosophila* is not monophyletic. Several genera , including *Engiscaptomyza*, *Hirtodrosophila*, *Mycodrosophila*, *Paramycodrosophila*, *Samoaia*, *Scaptomyza*, *Zaprionus*, and *Zygothrica* diverge within

the boundaries of what is currently considered the "genus" *Drosophila*. Furthermore, the subgenus *Drosophila* is not monophyletic and can be tentatively divided into at least two, and probably more, monophyletic groups. These clades correspond, although not perfectly, with the *virilis-repleta* and *immigrans-tripunctata* radiations described in earlier taxonomic studies. Finally, the subgenus *Sophophora*, previously considered the sister clade of the subgenus *Drosophila*, is shown to be a lineage distinct from the genus *Drosophila*.

CHAPTER 1

INTRODUCTION

1.1 Background

The family Drosophilidae contains approximately 3,000 species, distributed among over 60 genera (Wheeler 1982; 1986). Although some taxa within Drosophilidae have been widely studied with respect to their evolution (Patterson and Stone 1952; Powell 1997), ecology (Throckmorton 1975), genetics (Ashburner 1989; Lindsley and Zimm 1992) and development (Lawrence 1992), the phylogenetic relationships within this family as a whole are the subject of much debate (DeSalle and Grimaldi 1991; 1992). In many cases the evolutionary relationships within the family Drosophilidae are only based upon the original taxonomic descriptions of a limited number of type specimens, not strict phylogenetic analysis. Even when there has been extensive phylogenetic treatment of a given drosophilid group, the results of different studies often conflict with one another. Several phylogenetic studies have used either molecular sequence data (DeSalle 1992; Pelandakis and Solignac 1993; Russo *et al.* 1995) or morphological characters (Throckmorton 1975; Okada 1989; Grimaldi 1990) to assess relationships in Drosophilidae, but no study agrees completely with another.

The most recent morphological revision of Drosophilidae (Grimaldi 1990) used a cladistic analysis of over 200 morphological characters to divide Drosophilidae into tribes,

subtribes, infratribes, genus complexes, genus groups, and genus subgroups. In addition to these higher level changes, Grimaldi (1990) reorganized the genus *Drosophila* by removing several subgenera and elevating them to generic rank. Two subgenera, *Scaptodrosophila* (Duda 1923) and *Hirtodrosophila* (Duda 1923), were reclassified as basal genera within the subfamily Drosophilinae, not placed within the more derived genus *Drosophila*, as early taxonomic studies had suggested. The large radiation of *Drosophila* endemic to the Hawaiian Islands, or "Hawaiian *Drosophila*" (Kaneshiro 1974; 1976; Throckmorton 1966; 1975), forms a clade separate from what is traditionally recognized as the genus *Drosophila*. Grimaldi renamed this clade *Idiomyia* (Grimshaw 1901, *sensu* Grimaldi 1990) and placed them in the *Hirtodrosophila* genus complex, sister to the *Zygothrica* Genus Group, which contains the mycophageous genera *Hirtodrosophila*, *Mycodrosophila*, *Zygothrica*, *Paramycodrosophila*, and *Paraliodrosophila*. The Hawaiian radiation of flies traditionally placed in the genus *Scaptomyza*, "Hawaiian *Scaptomyza*" (Kaneshiro 1974; 1976; Throckmorton 1966; 1975), are not the sister group of the Hawaiian *Drosophila* in Grimaldi's (1990) phylogeny, but form a monophyletic clade with the remainder of the subgenera in *Scaptomyza*.

Although removing *Scaptodrosophila* from the genus *Drosophila* is congruent with all molecular analyses, Grimaldi's (1990) reorganization of the genus *Drosophila*, particularly with respect to the Hawaiian taxa, met with a great deal of opposition from both traditional taxonomists and molecular systematists, all of whom include the

Hawaiian Drosophilidae within the genus *Drosophila*. Furthermore, other genera, such as *Hirtodrosophila*, *Samoaia*, *Scaptomyza*, and *Zaprionus*, which were defined as being distinct from *Drosophila* (Grimaldi 1990), may actually evolve from within a polyphyletic *Drosophila* (Remsen and DeSalle 1998).

Reasons for incongruence between studies can be attributed to a variety of factors (Brower, *et al.* 1996). Poor species sampling in a study may result in many long branches, which can bias phylogenetic results (Felsenstein 1978). Selecting a sequence that is evolving too rapidly (or too slowly) for a given question may yield unresolved or incorrect results (Leconitre, *et al.* 1993). Another problem comes from basing the phylogenetic hypothesis of a group of species on a single gene sequence, which may accurately represent the history of the gene, but not necessarily the species being examined (Pamilio and Nei 1988; Hudson 1990). Finally, differences in the method used to reconstruct phylogenetic relationships can yield different phylogenies due to the ways in which the algorithms analyze the data (Hillis and Huelsenbeck 19??).

1.2 Explanation of Dissertation Format

The major chapters of this dissertation consist of a paper in press, two submitted manuscripts, and a manuscript to be submitted, each contained in a separate appendix. Appendix A, "Phylogeny of the *Drosophila saltans* species group based on combined

analysis of nuclear and mitochondrial DNA sequences," is in press and will appear in the June 1998 issue of *Molecular Biology and Evolution*. It is co-authored by J. B. Clark and M. G. Kidwell. The DNA sequences in this study were generated by myself and J. B. Clark, the writing is my own work. Appendix B, "Reevaluation of phylogeny in the *Drosophila obscura* species group based on combined analysis of nucleotide sequences," consists entirely of my own work and is submitted to *Molecular Phylogenetics and Evolution*. Appendix C, "Phylogeny of the subgenus *Sophophora* (Diptera:Drosophilidae) inferred using the 28S, alcohol dehydrogenase, and cytochrome oxidase II genes," is a collaboration between myself and M. G. Kidwell. The DNA sequencing, phylogenetic analysis, and writing is entirely my own. This manuscript has been submitted to *Systematic Entomology*. Appendix D, "Phylogenetic relationships of flies in the family Drosophilidae inferred from molecular and morphological characters," This manuscript is entirely my own work and is to be submitted to *Molecular Phylogenetics and Evolution*.

CHAPTER 2

PRESENT STUDY

My interests are in determining the phylogenetic relationships of flies within the family Drosophilidae. The primary focus of the analyses presented in this thesis is to determine phylogenetic relationships at four different taxonomic levels, within the species group, among species groups within a subgenus, and among genera within the Drosophilidae. However, the data gathered to accomplish this goal may also be used to explore issues involved in partitioning and combining data from different sources in phylogenetic analyses. Each study uses several molecular, and in some cases morphological, data sets to infer phylogenetic relationships. Individual and combined analyses are used to estimate phylogenetic relationships in all four studies.

Appendix A is a phylogeny of closely related species placed in the *Drosophila saltans* species group. I infer a phylogeny based on nucleotide sequences and compare it to the morphological hypothesis of relationships (Throckmorton and Magalhaes 1962) and determine that, although the morphological hypothesis is much less resolved, they are congruent with one another. I also address phylogenetic relationships within the recently diverged *saltans* subgroup. Previous work based on polytene chromosome banding patterns was incongruent with work based on reproductive isolation (Bicudo 1973a, b). The molecular characters are unable to resolve relationships within the *saltans* subgroup.

Appendix B is a reevaluation of phylogenetic relationships within the *Drosophila obscura* species group. Even though this group has been studied extensively, relationships among the five subgroups have proven to be enigmatic. My analyses do support the monophyly of the New World and Old World species groups, although this is weak. Surprisingly, some combined analyses also indicate that the Afrotropical *microlabis* subgroup may be the sister group of the *subobscura* subgroup. This relationship has not been seen before, but is consistent with biogeography (Throckmorton 1975).

Phylogenetic relationships within the subgenus *Sophophora*, members of which have been used as model systems in genetics, development and evolution for almost a century, are studied in Appendix C. My results indicate that *Sophophora* is monophyletic, despite the results of some previous analyses (Pelendakis, et al 1991). However, two species groups within *Sophophora*, the *melanogaster* and *willistoni*, are not resolved as monophyletic in this study. This may be due to a rapid bursts of speciation at the base of these clades. More intensive species sampling coupled with characters from additional nucleotide sequences and morphology are needed to address this issue.

Appendix D is an analysis of higher-level relationships within Drosophilidae. The placement of the Hawaiian Drosophilidae, which is controversial because of incongruent molecular and morphological hypotheses, was addressed. All Hawaiian taxa, including

members of the genera *Drosophila* and *Scaptomyza* are placed within the genus *Drosophila*. This is congruent with both the molecular data and a reanalysis of the morphological data. However, this genus *Drosophila* does not form a clade. The genera *Samoaia*, *Scaptomyza*, and *Zapriomus* (and possibly others) are nested within this group. Reclassification of this genus, and probably the entire family as well, perhaps employing a "phylogenetic taxonomy" approach should be undertaken.

The value of phylogenetic information is becoming increasingly appreciated. Several biological disciplines have begun to use the information contained in phylogenetic trees to better understand a variety of topics including development (Patel *et al.* 1994), behavior (de Queiroz and Wimberger 1993; de Queiroz in press), character evolution (Donoghue 1989; de Queiroz 1996) and the evolution of transposable elements (Clark *et al.* 1994). It has become clear that studying a group of organisms in the context of their evolutionary relationships is far more powerful than in the absence of this information. The phylogenies generated by this study can be used to examine the evolution of several interesting characters in the Drosophilidae, including the evolution of wing shape and patterning, broad headedness, and a variety of bristle morphologies and patterns (Powell and DeSalle 1995; Stark, *et al.* 1998). Better phylogenetic hypotheses for Drosophilidae, such as those presented in this thesis, will be powerful tools which can be used as a framework to analyze the evolutionary, genetic, developmental, and ecological information available for this group (Powell and DeSalle 1995).

CHAPTER 3

CONCLUSIONS

In this dissertation I have used phylogenetic methods to infer phylogenetic relationships at three taxonomic levels within the family Drosophilidae. Several important results of the papers that comprise this dissertation are:

1. Phylogenetic relationships within the *Drosophila saltans* species group based on molecular characters are congruent with, but better resolved than, those based on morphological characters.

2. The conflicting relationships of species within the *saltans* subgroup are not resolved with the use of molecular data.

3. The New World species subgroups (*affinis* and *pseudoobscura*) in the *Drosophila obscura* group are monophyletic.

4. The Old World species subgroups (*microlabis*, *obscura* and *subobscura*) in the *Drosophila obscura* group are monophyletic.

5. Based on some phylogenetic analyses and biogeographical information, the Afrotropical *microlabis* subgroup may be the sister clade of the *subobscura* subgroup.

6. The subgenus *Sophophora* is monophyletic with respect to the genus *Drosophila*.

7. The *obscura* and *melanogaster* species groups form a clade.

8. The *saltans* and *willistoni* species groups form a clade.

9. Within *Sophophora*, the *obscura* and *saltans* species groups are each monophyletic.

10. The *melanogaster* and *willistoni* species groups are not supported as clades in the *Sophophora* analyses. More extensive species sampling, perhaps coupled with additional character information, is needed to resolve these nodes.

11. The genus *Drosophila* is not a monophyletic group. Several genera are present within the bounds of what is currently recognized as the genus *Drosophila*.

12. The subgenus *Drosophila* is also not monophyletic. In these analyses this group is divided into two clades. These clades correspond roughly to the *virilis-repleta* and *immigrans-tripunctata* radiations described by Throckmorton (1975).

13. The subgenus *Sophophora* is not the sister group of the subgenus *Drosophila*. *Sophophora* represents a distinct lineage from the genus *Drosophila*.

14. Simultaneous phylogenetic analysis, coupled with individual analyses, is a more powerful approach to determining phylogenetic relationships

than either is alone.

15. Measures of support, such as partitioned branch support and hidden support can more clearly define interactions among data sets in simultaneous analysis, making is easier to determine which data partition is lending support at any given node.

This dissertation examines the phylogenetic relationships of flies within the family Drosophilidae at several taxonomic levels. Seven data partitions, six molecular and one morphological, are analyzed in both individual and combined analyses. Bootstrap proportions and decay indices are used to measure support in all analyses. Several measures of support specific to combined analysis, such as partitioned branch support (Baker and DeSalle 1997; Gatesy, *et al.* 1998) and hidden support (Gatesy, et al. 1998) are also employed in these analyses to examine contributions of separate data partitions in simultaneous analysis.

Even though this thesis adds much to our understanding of evolution of flies within Drosophilidae, much work remains to be done. The molecular data sets presented in the genus level study, particularly *Gpdh* and *Sod*, are not nearly as comprehensive in species sampling as the morphological data set (Grimaldi 1990). Additional field collections in Latin America, southeast Asia, and tropical Africa must be made increase sampling. These collections should be accompanied by sequencing of the loci presented

here, as well as several others, to better resolve phylogenetic relationships among the major clades of the family Drosophilidae. This expanded phylogeny will eventually be used to redefine higher taxonomic groups (subgenera, genera, and tribes) within this family. These data will also be used, in conjunction with several drosophilid fossils, to date major points of divergence within this family.

Other studies, beyond basic phylogenetic taxonomy should also be undertaken. The value of phylogenetic information in many different biological disciplines is now becoming appreciated. The phylogenetic information presented here can be applied, for example, to studies of development to further our understanding of the genetic bases of morphological change over time.

APPENDIX A

PHYLOGENY OF THE *DROSOPHILA SALTANS* SPECIES GROUP BASED ON COMBINED ANALYSIS OF NUCLEAR AND MITOCHONDRIAL DNA SEQUENCES

Phylogeny of the *Drosophila saltans* species group based on combined analysis of nuclear and mitochondrial DNA sequences

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Running Head: *Drosophila saltans* phylogeny

Key Words: alcohol dehydrogenase, cytochrome oxidase I, cytochrome oxidase II, ITS1, taxonomic and character congruence, *Drosophila saltans* phylogeny

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Abstract

Nucleotide sequences from two nuclear loci, alcohol dehydrogenase and the Internal Transcribed Spacer-1 of the nuclear ribosomal DNA repeats, and two mitochondrial genes, cytochrome oxidase I and cytochrome oxidase II, were determined from nine species in the *Drosophila saltans* species group. The partition homogeneity test and partitioned Bremer support were used to measure incongruence between phylogenetic hypotheses generated from individual partitions. Individual loci were generally congruent with each other and consistent with the previously proposed morphological hypothesis, though they differed in level of resolution. Since extreme conflict between partitions did not exist, the data were combined and analyzed simultaneously. The phylogeny inferred from the total evidence method gave a more resolved and highly supported phylogeny, as indicated by bootstrap proportions and decay indices, than any of the individual analyses. The *cordata* and *elliptica* subgroups, considered to have diverged early in the history of the *D. saltans* group (Throckmorton 1975), were sister taxa to the remainder of the *saltans* group. The *sturtevanti* subgroup, represented by *D. milleri* and *D. sturtevanti*, occupies an intermediate position in this phylogeny. The *saltans* and *parasaltans* subgroups are sister clades and occupy the most recently derived portion of the phylogeny. As with previous morphological studies, phylogenetic relationships within the *saltans* subgroup were not satisfactorily resolved by the molecular data.

Introduction

The *Drosophila saltans* group is one of four major species groups placed in the subgenus *Sophophora* (Sturtevant 1942). Throckmorton (1975) considered the neotropical *saltans* and *willistoni* species groups to be distinct and derivative lineages within *Sophophora*, clearly separated from the Old World *melanogaster* and *obscura* species groups. The *saltans* species group consists of 21 species which are divided into five subgroups; *cordata*, *elliptica*, *parasaltans*, *saltans*, and *sturtevanti* (table 1) on the basis of a variety of morphological characters (Magalhaes and Bjornberg 1957; Magalhaes 1962; Throckmorton and Magalhaes 1962).

Based on contemporary distribution patterns and geological information, Throckmorton (1975) proposed that the ancestor of the *saltans* species group originated in tropical North America where the so-called "primitive" *cordata* and *elliptica* subgroups are found. This ancestral group colonized the South American continent and the *sturtevanti*, *saltans*, and *parasaltans* subgroups (the "derived" *saltans* subgroups) then diversified prior to the formation of the present day isthmus of Panama. Some members of the *saltans* subgroup, such as *D. saltans* and *D. prosaltans*, have recently diffused back into North America, probably within the past 4.5 million years (Throckmorton 1975). Within the *saltans* subgroup, species-level relationships are unresolved because of

the short time since divergence and conflict between reproductive isolation studies and the chromosome inversion phylogeny (Bicudo 1973 a,b).

This study uses four molecular loci (*Adh*, *COI*, *COII*, and ITS1) as well as a morphological data set (Magalhaes 1962) to examine the phylogeny of the *Drosophila saltans* species group. We are interested in estimating the phylogeny of the five major lineages in the *saltans* species group and the species-level relationships within the *saltans* subgroup.

When two or more data partitions are examined in separate phylogenetic analyses, the resultant tree topologies often do not completely agree with one another, or with the combined data set (Chippendale and Weins 1994). There are several schools of thought concerning how data partitions, which may be more or less incongruent with one another, should be analyzed (reviewed in deQueiroz, *et al.* 1995; Brower, *et al.* 1996). One method is taxonomic congruence, where agreement among well-supported topologies derived from separate analyses of different data sets is presented as a consensus tree (Mikevich 1978; Miyamoto and Fitch 1995). Another method is character congruence, or total evidence (Kluge 1989). In this approach, all data are combined and analyzed simultaneously to increase descriptive efficiency and explanatory power of the data (Kluge 1989; Barrett, et al 1991; Eernisse and Kluge 1993; Jones, et al 1993; Kluge and Wolf 1993). A compromise between taxonomic congruence and total evidence, referred to as conditional data combination, or prior agreement, has been proposed by several

systematists (de Queiroz 1993; Bull, et al 1993; Huelsenbeck, et al 1996). This begins with an analysis of separate data sets, termed process partitions (Bull, et al 1993), followed by a test for heterogeneity between partitions. If significant between-partition heterogeneity does not exist, the data are combined and analyzed simultaneously.

Several methods to test for heterogeneity between data sets have been proposed (Farris, et al. 1994, 1995; Huelsenbeck and Bull 1996; Baker and DeSalle 1997). Our work on the *Drosophila saltans* species group offers an opportunity to compare several of these measures of heterogeneity and to assess their implications for the methods of taxonomic congruence, total evidence and prior agreement in reconstructing the phylogeny of the *Drosophila saltans* species group.

Materials and Methods

DNA Sources

Live *Drosophila* stocks were obtained from the National *Drosophila* Species Resource Center in Bowling Green, Ohio. Table 1 shows the taxonomic classification of the sixteen lines used in this study and where each was collected. The following sequences were obtained from the literature: *D. melanogaster* X78384 (*Adh*), J01404 (*COI* and *COII*), M21017 (ITS1); *D. yakuba* X54120 (*Adh*), X03240 (*COI* and *COII*), Z28416 (ITS1). Genbank accession numbers for sequences determined as a result of this

study are: AF045081-AF045096 (COII), AF045097-AF045112 (COI), AF045113-AF045126 (Adh); AF045363-AF045371 (ITS1).

Sample Preparation and DNA Sequencing

Genomic DNA was isolated following the method of Gloor and Engels (1992). The four target loci were amplified from each taxon using standard PCR cycling conditions. PCR primers were designed based on the previous studies, referred to in table 2. PCR products from the 305 bp fragment of the *COI* gene and the entire *COII* gene (688 bp) were purified by membrane filtration (Millipore) and sequenced directly using a standard dsDNA cycle sequencing protocol (GIBCO-BRL). PCR products from the entire coding region of the *Adh* gene (771 bp) and the entire ITS1 locus (785 aligned positions) were cloned into the TA cloning vector (Invitrogen) or the PCR-SCript vector (Stratagene). Single colonies were selected and sequenced using either a dsDNA cycle sequencing procedure (GIBCO-BRL) or the Sequenase sequencing kit (Amersham). Nucleotide sequences were determined from between 80 and 100 percent of both strands of the *Adh*, *COI*, and *COII* genes. Multiple clones from each species were obtained for the ITS1 locus and the nucleotide sequence of one strand of each clone was determined. Where discrepancies existed between clones from the same species, the differences were verified by consulting the original autoradiograms.

Sequence Alignment

The *COI* and *Adh* coding regions required no gaps to align the species in this study. The *COII* gene required the inclusion of a single gap (positions 673-675) in the outgroup species to align with the *saltans* species group. Any gaps in these analyses were treated as missing data. The non-coding nature of the ITS1 region and notable size variation between species, optimal alignment of this locus was achieved only with the use of appropriate gaps. Clustal W (Thomson, Higgins, and Gibson 1994) and Malign, version 2.1 (Wheeler and Gladstein 1994), were used to obtain an optimal alignment of the ITS1 region. The phylogenetic relationships between taxa remained the same when ITS1 was analyzed with and without the gapped positions.

Phylogenetic Analysis of Nucleotide Sequences

All analyses described below were performed using a variety of optimality criteria, including maximum likelihood (ML), neighbor joining (NJ) and maximum parsimony (MP), to estimate the phylogeny of the *saltans* species group. We present only the MP analyses. All nucleotide partitions were examined both individually and in simultaneous analyses. A variety of weighting schemes (transversions 2X over transitions, transversions 4X over transitions, transversions only) were employed and all gave results congruent with one another. Here we present unweighted parsimony searches which use the branch and bound algorithm implemented in PAUP 4.0d54

(Swofford 1997). Table 2 shows some important aspects of each analysis performed. The level of confidence in each node of all most parsimonious trees obtained was assessed using bootstrap proportions (Felsenstein 1985, 1988) and decay indices (Bremer 1988; Donoghue *et al.* 1992). All trees presented are 50% majority-rule consensus phylogenies resulting from 200 bootstrap replicates. Bootstrap proportions are shown above the node and decay indices are below the node in each tree. All trees are rooted using two members of the *melanogaster* species group, *D. melanogaster* and *D. yakuba*. MacClade, ver 3.0 (Maddison and Maddison 1992), was used for a variety of phylogeny manipulations and character state analyses.

Phylogenetic Analysis of Morphological Data

The morphological data set used in this study was adapted from morphological characters used by Magalhaes (1962). The characters examined include: the presence/absence of mesonotal pattern, the presence/absence of subcarinal hairs, a dark *versus* yellow body color, the presence/absence of sensilla on fist sternite, the presence/absence of sensilla in seventh sternite of males, and the presence/absence/reduction of vestigial plates of first sternite of both males and females. Five continuous characters used by Magalhaes (1962) were omitted because it was difficult to code these characters for parsimony analysis. MP analyses were preformed

on these data individually and in combination with the nucleotide data (see Table 2). The morphological characters were not used in maximum likelihood or distance analyses.

Phylogenetic Tree Comparisons

We used the partition homogeneity test (as implemented in PAUP 4.0d54; Swofford 1997) to examine differences (1) between each locus and (2) between each locus and the total evidence hypothesis (table 3). We also used partitioned Bremer support (Bremer 1988; 1992; Baker and DeSalle 1997) to measure the amount of support provided by each partition to each node on the total evidence phylogeny.

Partitioned Bremer support (PBS) shows the contribution of each partition to the decay index of every node on the total evidence tree (Baker and DeSalle 1997). To obtain the PBS value for a given node on the total evidence tree, the length of the partition on the unconstrained total evidence tree is subtracted from the length of a partition on a tree constrained to contain only the node of interest. If the partition supports a relationship represented by a node in the total evidence tree, the constraint tree will be longer and the the PBS value will be positive. If, on the other hand, a partition supports an alternative relationship, the constraint tree will be shorter and the PBS value will be negative, indicating incongruence with the simultaneous analysis. The magnitude of PBS values indicate the level of support for, or incongruence with, a node (Baker and DeSalle 1997). The sum of all partition lengths for any given node will always sum to the decay index for

that node on the total evidence tree. Using this method allows us to determine the relative contribution of each partition to the simultaneous analysis tree (table 4).

Results

Phylogenetic Relationships -- *Adh*

Figure 1A shows the phylogenetic hypothesis for the *saltans* species group based on the alcohol dehydrogenase gene (see also table 2). This locus was unable to resolve the branching order among any of the five species subgroups. Furthermore, the relationships among the recently diverged species of the *saltans* subgroup were completely unresolved. However, the *Adh* sequence was able to resolve the relationship between *D. milleri* and *D. sturtevanti* in the *sturtevanti* species subgroup (fig. 1A, clade E) and between the various geographic isolates of *D. emarginata* (fig. 1A, clade B).

Phylogenetic Relationships -- ITS1

A phylogeny of the *saltans* species group, based on the ITS1 locus, is presented in figure 1B (see also table 2). The ITS1 locus places the *cordata* subgroup (fig. 1B, clade A) as a sister taxon to the *elliptica* subgroup (fig. 1B, clade B), consistent with morphological studies (Magalhaes 1962). This locus is also able to resolve some relationships among species in the *saltans* (fig. 1B, clade D) and *sturtevanti* (fig. 1B, clade E) subgroups.

However, this sequence provides no information concerning the phylogenetic relationships among most of the subgroups in the *saltans* species group.

Phylogenetic Relationships -- *COI*

Figure 2A shows a phylogeny based on the mitochondrial cytochrome oxidase I gene. This tree shows much more structure than either the *Adh* or ITS1 trees. It shows that the "derived" (*sensu* Throckmorton 1975) members of the *saltans* group (the *parasaltans*, *saltans*, and *sturtevanti* subgroups) are monophyletic. However, the phylogeny cannot reliably determine whether the *parasaltans* or *sturtevanti* subgroup (fig. 2A, clades C and E) is the sister taxon of the *saltans* subgroup (fig. 2A, clade D). Within the *saltans* subgroup (fig. 2A, clade D), *D. lusaltans* is shown to be the sister taxon to the remainder of the *saltans* subgroup. Interestingly, the *D. prosaltans "Costa Rica"* is the sister taxon to *D. austrosaltans*, to the exclusion of *D. prosaltans "Colombia."* This result is incongruent with both reproductive isolation and chromosome inversion studies (Bicudo 1973 a,b). The *cordata* and *elliptica* subgroups (fig. 2A, clades A and B) are placed at the base of the *saltans* species group, in agreement with previous morphological work (Throckmorton and Magalhaes 1962). However, this locus is unable to resolve the deeper branching nodes in the phylogeny.

Phylogenetic Relationships -- *COII*

Figure 2B shows the mitochondrial cytochrome oxidase II phylogeny (see also table 2). All sequences from the *saltans* species group are distinguished from the outgroup sequences by a single three base pair deletion located at the 3' end of the sequence in all *melanogaster* group species. While this mitochondrial locus gives more phylogenetic resolution than the *Adh* or ITS1 sequences, it is not able to resolve the branching order among the *cordata*, *elliptica*, *saltans* and *sturtevanti* subgroups (fig. 2B, clades A, B, D and E). However, within these subgroups, phylogenetic relationships are congruent with the other loci in this study and with the previous morphological work. *D. austrosaltans* is shown to be the sister taxon to the remainder of the *saltans* subgroup (fig. 2B, clade D), a placement which is consistent with reproductive isolation studies (Bicudo 1973 a). *D. lusaltans* is the next species to branch off from this lineage, possibly when it colonized the Caribbean Islands. The closely related species *D. saltans* and *D. prosaltans* form a sibling species cluster. The *COII* phylogeny places the *parasaltans* subgroup (fig. 2B, clade C) at the base of the *saltans* phylogeny. Although this placement is congruent with the ITS1 phylogeny (fig. 1B), it is incongruent with the *Adh* and *COI* gene trees (figs. 1A and 2A), and with the traditional view of phylogeny in this group (Throckmorton and Magalhaes 1962).

Phylogenetic Relationships -- Morphology

The morphological data set contained eight characters dealing with characters such as body color and pattern, bristle number, and the shapes of a variety of other structures. Only one geographic isolate for each species is analyzed in the original paper (Magalhaes 1962). Furthermore, all taxa not available for nucleotide saquencing were omitted from this search. There is a single most parsimonious tree (table 2) when the morphological data is analyzed phylogenetically (phylogeny not shown). This data places the *elliptica* and *cordata* subgroups as sister taxa, with the *parasaltans* subgroup being the sister group of the *elliptica-cordata* clade. The *saltans* and *sturtevanti* subgroups as unresolved with respect to one another. They are placed sister to the *elliptica-cordata-parasaltans* clade.

Comparisons Among Data Sets

Table 3 shows the results of the partition homogeneity test. Pairwise comparisons which show significant homogeneity ($p<0.10$) are indicated. The ITS1 and morphological data sets stand out as being incongruent with most, but not all, of the other partitions in this study. For example, ITS1 shows significant heterogeneity when compared to the morphological and mitochondrial partitions, but not to the other nuclear partition, *Adh*. The morphological partition is incongruent with ITS1 and *COII*, but not *COI* or *Adh*.

PBS values were calculated for all nodes, numbered 1-10, on the total evidence tree (table 4). The morphological data set was incongruent with node 7, the *saltans-parasaltans* subgroup relationship (fig. 3), and instead supports grouping the *parasaltans*, *cordata*, and *elliptica* subgroups in a clade. The alcohol dehydrogenase partition was mostly congruent with the total evidence hypothesis, supporting seven and being equivocal at two of ten nodes. *Adh* conflicted with the total evidence tree only at node 2, which united the Central American populations of *D. emarginata* to the exclusion of the isolate from Ecuador. The ITS1 locus was in agreement with three of the ten nodes and equivocal at half of the nodes on the simultaneous analysis tree. The ITS1 data disagreed at nodes 3 and 10, probably because of lack of resolution present in the individual analysis. The *COI* partition supported seven of ten nodes on the total evidence tree. but was incongruent at two places, node 1 and node 7. The *COII* gene was the partition most congruent with the total evidence hypothesis supporting nine of the ten nodes on the total evidence tree. Only node 8, which supports the "derived" saltans clade (Throckmorton 1975), was shown to be incongruent.

Phylogenetic Relationships -- Total Evidence Analysis

The total evidence phylogeny (fig. 3) includes the morphological data set of Magalhaes (1962) and all four molecular data sets generated in this study (table 2). This phylogeny places the *parasaltans* and *saltans* subgroups as sister taxa (fig. 3, clades C

and D). Within the *saltans* subgroup, which has diversified only recently, relationships are mostly unresolved. This is probably due to lack of informative sites and conflicting information from the different sequences used in this study. The *sturtevanti* subgroup (fig. 3, clade E) is the sister taxon to the *saltans-parasaltans* clade. The *cordata* and *elliptica* subgroups (fig. 3, clades A and B) are sister to the "derived" *saltans* subgroups, with the *cordata* subgroup representative, *D. neocordata*, being the sister taxon to all other *saltans* group species. The analyses are in agreement with the previous taxonomic work on the *saltans* species group (Magalhaes 1962, Throckmorton 1975). However, the molecular data are unable to resolve the most difficult systematic issue, the branching order within the *saltans* subgroup.

Table 5 shows the partitions that, when analyzed individually, unequivocally support nodes seen in the total evidence tree. Several monophyletic groups are present in all partitions, including those represented by nodes 3, 4, and 5. Interestingly, two nodes that are present in the total evidence tree, 7 and 9, are absent in all individual partition analyses, indicating that these partitions either lack the resolution of the total evidence tree or support an alternative relationship.

Discussion

Comparisons of Phylogenetic Hypotheses

Visual inspection of the phylogenies derived from each partition (figs. 1,2) indicates that they differ in (1) their placement of the *parasaltans* subgroup and (2) the branching order within the *saltans* subgroup. The partition homogeneity test does in fact show the morphological and ITS1 data to be incongruent with some other partitions in this study. However, each of these partitions are congruent with at least one other partition. For example, this test cannot reject homogeneity when comparing the ITS1 partition to the *Adh* partition or the morphological partition to either the *COI* or *Adh* partitions. Therefore, no partition is in conflict with all other partitions. Furthermore, all partitions contribute to PBS values (table 4), indicating that each partition does have influence on the topology of the total evidence tree. Therefore, if one were employing a prior agreement approach, it would be difficult to determine which data partition to exclude from the analysis. Baker and DeSalle (1997) encountered this same problem in their study of phylogeny in the Hawaiian *Drosophila*. They concluded that if a partition was homogeneous when compared to at least one other partition, then it should be included in the total evidence analysis. We agree with this conclusion and propose that all partitions in this study be combined in a simultaneous analysis to estimate the *saltans* group phylogeny.

Individual analyses indicate that the different data partitions are incongruent in the placement of the *parasaltans* subgroup, represented by *D. subsaltans*. The *Adh* and ITS1 partitions do not yield any information on the relationships of this taxon to any of the

other subgroups. The *COI* partition indicates that *D. subsaltans* is closely related to the *saltans* and *sturtevanti* subgroups, although it is unclear which subgroup is most closely related. The *COII* partition shows weak support for this subgroup being the sister taxon to all other *saltans* species. However, the partition homogeneity test is unable to reject the null hypothesis of homogeneous data for comparisons between the *COI* and *COII* partitions (table 3). Therefore, it would seem that, although the relationships presented in the *COI* and *COII* bootstrap trees are in conflict, this conflict is not statistically significant.

The simultaneous analysis indicates support for a *saltans-parasaltans* clade (fig. 3, node 7), a relationship not seen in any individual analysis. Examining the PBS values (table 3) shows that the ITS1 and *COII* partitions support this relationship and that *COI* and the morphological partitions support alternative relationships. This result is somewhat surprising since the ITS1 and *COII* partitions alone did not support a *saltans-parasaltans* clade. However, previous studies have demonstrated that combined analyses can uncover phylogenetic affiliations not observed in individual analyses (Chippendale and Weins 1994). It is possible that, in the simultaneous analysis, character conflict present in individual partitions is resolved to support the *saltans-parasaltans* clade.

There are also conflicts between partitions when estimating the phylogeny of the *saltans* subgroup. No two gene trees give the same branching order within the *saltans*

subgroup and some partitions, such as *Adh*, yield no information concerning these relationships at all. The *COI* partition is incongruent with all other partitions in that it shows *Drosophila prosaltans* to be paraphyletic with respect to *Drosophila austrosaltans*. The ITS1 and *COI* partitions place *D. austrosaltans* well within the *saltans* subgroup, while the *COII* partition places this species as a sister taxon to all species within this subgroup. Given the recent time of divergence (Throckmorton 1975), large population sizes (Throckmorton 1975), and potential for gene flow between these species (Bicudo 1973a) this conflict is not surprising. It is possible that ancestral polymorphisms are incompletely sorted within this subgroup, creating either a lack of resolution or conflict between different partitions.

Phylogeny of the *Drosophila saltans* Species Group

The total evidence tree (fig. 3) is in complete agreement with the proposed morphological phylogeny of the *saltans* group (Magalhaes 1962). Even the *saltans-parasaltans* relationship, which was not clearly seen in the separate molecular analyses, is resolved through the use of the total evidence method. The total evidence analysis shows all of the species subgroups to be monophyletic with respect to each other and to the outgroup species. The *cordata* subgroup, represented by *D. neocordata*, is the sister taxon to the rest of the species in the *saltans* group. The *elliptica* group is the next most basal subgroup. The molecular data agree with the morphological and biogeographical

studies (Magalhaes 1962; Throckmorton 1975) and place the *sturtevanti* subgroup at an intermediate position as the sister group to the *saltans* and *parasaltans* subgroups. It is interesting to note, however, that the total evidence phylogeny is not congruent with the phylogenetic re-analysis of a selected group of morphological characters. This is likely due to the fact that the taxonomists who established the various *saltans* subgroups took into account more discrete and continuous characters than were presented in Magalhaes (1962) and likely had a good "gestalt" feeling for how the groups were related based on fieldwork, biogeography, and laboratory experiments. The branching order within the *saltans* subgroup is not well defined because of the relatively recent divergence of these species and conflicting information from each locus. The molecular data are, therefore, unable to resolve the previous conflict between the results of reproductive isolation studies and the observations on chromosome inversion patterns (Bicudo 1973 a,b). We argue that, in the absence of more conclusive data, the phylogenetic relationships of species within the *saltans* subgroup should be presented as unresolved.

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Table 1

Taxonomic relationships and collection localities for species in the *Drosophila saltans* group.

Subgroup	Species	Collection Location	BG Stock Center Number
A. *cordata*	*neocordata*	Minas Gerias, Brazil	14041-0831.0
B. *elliptica*	*emarginata*	Turrialba, Costa Rica	14042-0841.0#
		La Palma, El Salvador	14042-0841.4
		Quito, Ecuador	14042-0841.7
C. *parasaltans*	*subsaltans*	Balem, Brazil	14044-0872.0
D. *saltans*	*austrosaltans*	Pirassununga, Brazil	14045-0881.0
	lusaltans	Petionville, Haiti	14045-0891.0
	prosaltans	Turrialba, Costa Rica	14045-0901.0#
		Leticia, Colombia	14045-0901.4#
	saltans	San Jose, Costa Rica	14045-0911.0
E. *sturtevanti*	*milleri*	El Yunque, Puerto Rico	14043-0861.0
	sturtevanti	Turrialba, Costa Rica	14043-0871.0#
		Volcan Soufriere, Lesser Antilles	14043-0871.2#
		Martinique, West Indies	14043-0871.9
outgroups	*melanogaster*	see materials and methods	
	yakuba	see materials and methods	

Table 2

Summary of results from maximum parsimony analyses.

Locus	Size[a]	PI[b]	# MPTs[c]	TL[d]	CI[e]	RI[f]	References[g]
COI	305	72	5	191	0.670	0.703	Simon, *et al.* 1994
COII	688	107	1	341	0.657	0.655	Beckenbach , Wei, and Liu 199
Adh	771	98	2	238	0.840	0.822	Russo, Takezaki and Nei 1995
ITS-1[h]	785	222	6	659	0.898	0.881	Vogler and Desalle 1994
Morph	7	5	4	8	0.875	0.875	Magalhaes 1962
mtDNA[i]	993	179	1	541	0.649	0.655	
nucDNA[j]	1556	316	1	898	0.881	0.834	
TE[k]	2549	499	6	1466	0.785	0.740	

[a] Size of locus (in base pairs)

[b] Number of parsimony informative sites

[c] Number of most parsimonious tree(s) recovered

[d] Tree length of most parsimonious trees

[e] Ensemble Consistency Index (Kluge and Farris 1969)

[f] Ensemble Retention Index (Archie 1989a,b; Farris 1989)

[g] Selected references used for primer design

[h] This analysis was performed with fewer taxa than the other individual-locus searches

[i] Combined mitochondrial analysis (*COI* + *COII*)

[j] Combined nuclear analysis (*Adh* + ITS-1)

[k] Total evidence tree

Table 3

Results of Partition Homogeneity Test.

	morph.	*COI*	*COII*	*Adh*	ITS-1	TE
morph.	–	1.0	0.09*	0.12	0.03*	0.04*
COI		–	0.12	0.77	0.02*	0.97
COII			–	0.31	0.09*	0.33
Adh				–	0.46	0.90
ITS-1					–	0.08*
TE						–

* Data partitions which display significant homogeneity when compared

Table 4

Results of Partitioned Bremer Support Analyses.

node	morphology	*COI*	*COII*	*Adh*	ITS-1	TE
1	0	-5.33	2.5	2.33	1.5	1
2	0	10	8	-1	0	17
3	0	5.5	3	1	-2.5	7
4	0	2	9	9	0	20
5	0	10.5	4.5	11	0	26
6	0	6	3.5	0	1.5	11
7	-1.5	-1	1.5	0	5	4
8	0	5	-4	5	0	6
9	0	0	1	2	0	3
10	0	5	19.5	62	-1.5	85
totals	-1.5	37.67	47.5	91.33	4	179

Table 5

Number of node on total evidence tree, monophyletic group that it represents and partition that supports that monophyletic group in individual analysis.

node	monophyletic group	partition with this clade
1	*D. prosaltans*	*COII, Adh*
2	Central American *D. emarginata*	*COI, COII*
3	*D. sturtevanti*	*COI, COII, Adh*, ITS-1
4	*D. emarginata/elliptica* subgrp.	*COI, COII, Adh*, ITS-1
5	*sturtevanti* subgrp.	*COI, COII, Adh*, ITS-1
6	*saltans* subgrp.	*COI, COII*, ITS-1
7	*saltans/parasaltans* subgrps.	--
8	"derived" *saltans*	*COI*
9	"dervied" *saltans* + *elliptica* subgrp.	--
10	all *saltans* species	*COI, COII, Adh*, ITS-1

Figure Legends

FIG. 1.– (A) The majority-rule bootstrap phylogeny based on the coding regions of the *Adh* gene. (B) The majority-rule bootstrap phylogeny based on the ITS1 region. Bootstrap proportions (above) and decay indices (below) are shown at each node. A = *cordata* subgroup, B= *elliptica* subgroup, C= *parasaltans* subgroup, D= *saltans* subgroup, E= *sturtevanti* subgroup.

FIG. 2.–. (A) The majority-rule bootstrap phylogeny based on a 305bp fragment of the mitochondrial *COI* gene. (B) The majority-rule bootstrap phylogeny based on the complete *COII* gene. Bootstrap proportions (above) and decay indices (below) are shown at each node. A = *cordata* subgroup, B= *elliptica* subgroup, C= *parasaltans* subgroup, D= *saltans* subgroup, E= *sturtevanti* subgroup.

FIG. 3.– The majority-rule bootstrap phylogeny based on total evidence analysis. Bootstrap proportions (above) and decay indices (below) are shown at each node. A= *cordata* subgroup, B= *elliptica* subgroup, C= *parasaltans* subgroup, D= *saltans* subgroup, E= *sturtevanti* subgroup. Numbered nodes, 1-10, correspond to table 5.

Figure 1

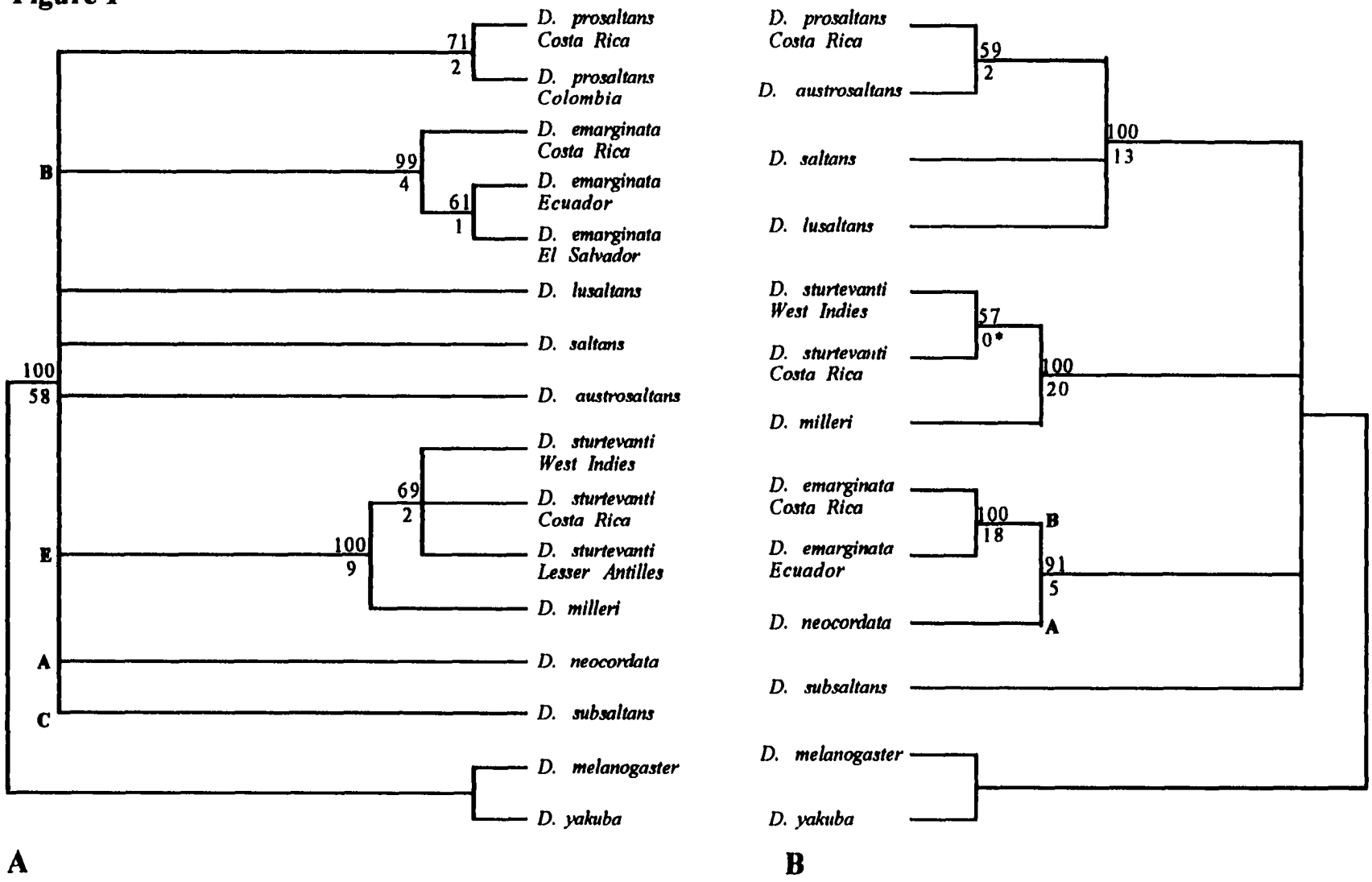

D. prosaltans Costa Rica
D. prosaltans Colombia
D. emarginata Costa Rica
D. emarginata Ecuador
D. emarginata El Salvador
D. lusaltans
D. saltans
D. austrosaltans
D. sturtevanti West Indies
D. sturtevanti Costa Rica
D. sturtevanti Lesser Antilles
D. milleri
D. neocordata
D. subsaltans
D. melanogaster
D. yakuba
71
2
99
4
61
1
100
58
69
2
100
9
B
E
A
C
A
D. prosaltans Costa Rica
D. austrosaltans
59
2
D. saltans
D. lusaltans
100
13
D. sturtevanti West Indies
D. sturtevanti Costa Rica
57
0*
D. milleri
100
20
D. emarginata Costa Rica
D. emarginata Ecuador
100
18
B
91
5
A
D. neocordata
D. subsaltans
D. melanogaster
D. yakuba
B

Figure 2

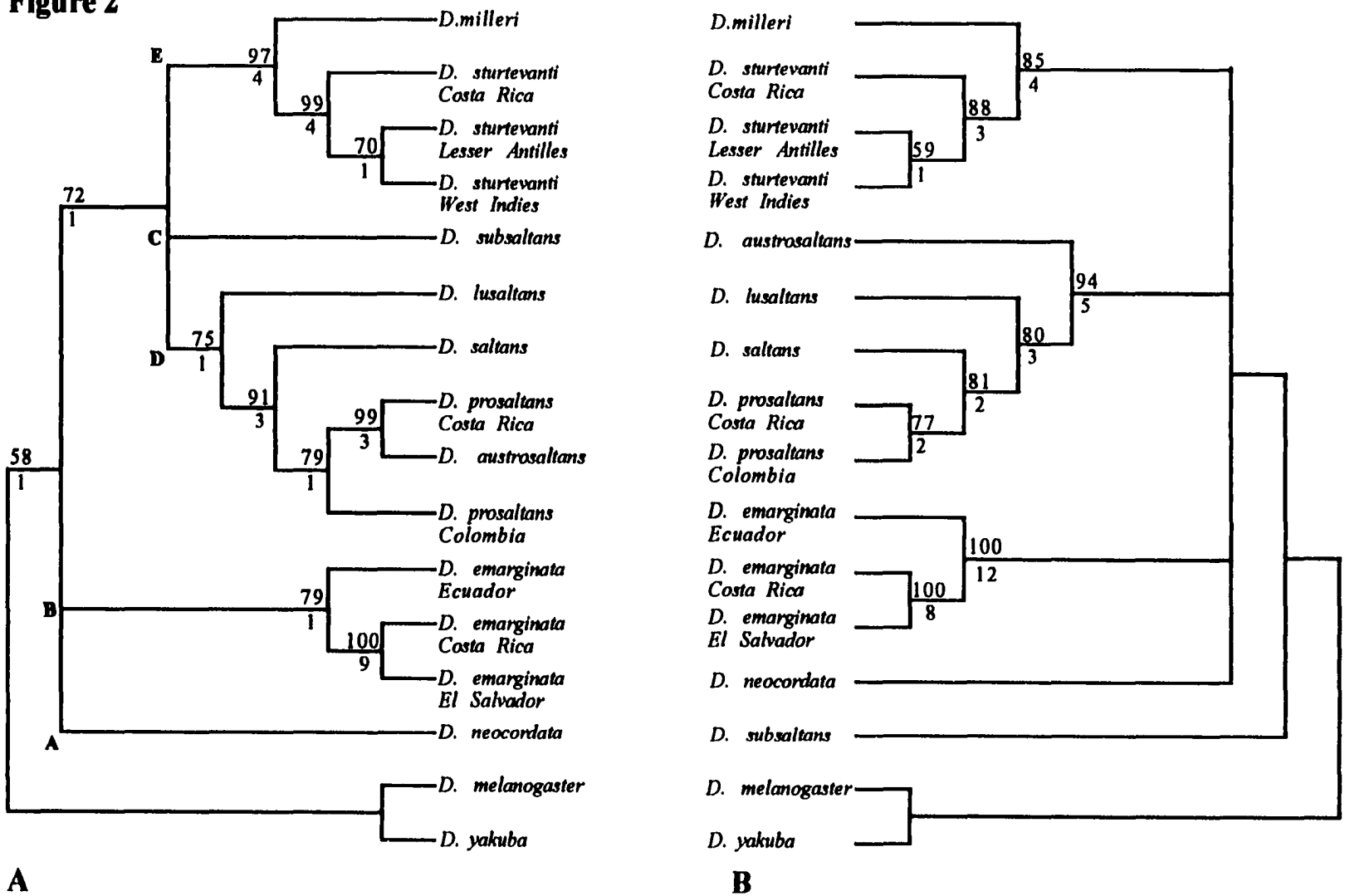
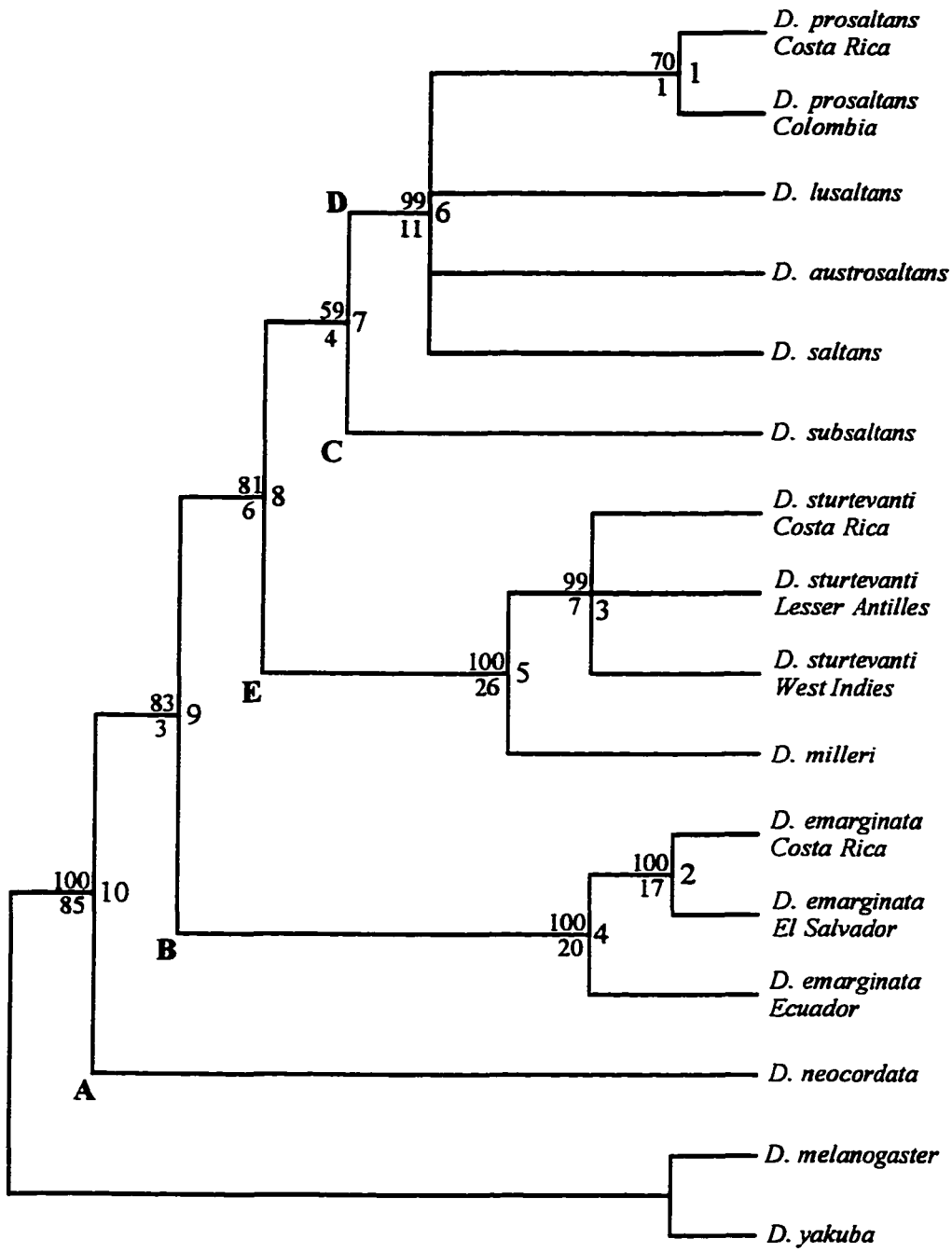

D.milleri
D. sturtevanti Costa Rica
D. sturtevanti Lesser Antilles
D. sturtevanti West Indies
D. subsaltans
D. lusaltans
D. saltans
D. prosaltans Costa Rica
D. austrosaltans
D. prosaltans Colombia
D. emarginata Ecuador
D. emarginata Costa Rica
D. emarginata El Salvador
D. neocordata
D. melanogaster
D. yakuba
A
B

Figure 3


D. prosaltans Costa Rica
D. prosaltans Colombia
D. lusaltans
D. austrosaltans
D. saltans
D. subsaltans
D. sturtevanti Costa Rica
D. sturtevanti Lesser Antilles
D. sturtevanti West Indies
D. milleri
D. emarginata Costa Rica
D. emarginata El Salvador
D. emarginata Ecuador
D. neocordata
D. melanogaster
D. yakuba
A
B
C
D
E
70
1
1
99
11
6
59
4
7
81
6
8
99
7
3
100
26
5
83
3
9
100
17
2
100
20
4
100
85
10

APPENDIX B

REEVALUATION OF PHYLOGENY IN THE *DROSOPHILA OBSCURA* SPECIES GROUP BASED ON COMBINED ANALYSIS OF NUCLEOTIDE SEQUENCES

Reevaluation of phylogeny in the *Drosophila obscura* species group based on combined analysis of nucleotide sequences.

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Running Head: *D. obscura* phylogeny

Key Words: Drosophila obscura species group, phylogeny, simultaneous analysis

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Abstract

The *Drosophila obscura* species group has served as an important model system in many evolutionary and population genetic studies. Despite the amount of study this group has received, some phylogenetic relationships remain unclear. While individual analysis of different nuclear, mitochondrial, allozyme, restriction fragment and morphological data partitions are able to discern relationships among closely related species, they are unable to resolve relationships among the five *obscura* species subgroups. A combined analysis of several nucleotide data sets is able to provide resolution and support for some nodes not seen or well supported in analyses of individual loci. Here we present a phylogeny of the *obscura* species group based on combined analysis of nucleotide sequences from six mitochondrial (mt) and five nuclear (nu) loci. The results of several different combined analyses indicate that the Old World *obscura* and *subobscura* subgroups form a monophyletic clade, although they are unable to resolve the relationships among the major lineages within the *obscura* species group.

Introduction

The *Drosophila obscura* species group has served as a model system for evolutionary studies for over 60 years (Dobzhansky and Powell 1975; Powell 1997). Members of this species group have been used to study the mechanisms of speciation (*e.g.*, Noor 1995), population genetics of polytene chromosome inversions (Popadic and Anderson 1994; Powell 1992), and phylogeny (*e.g.*, Barrio and Ayala 1997).

Sturtevant (1942) originally divided the *obscura* species group into two subgroups; the *affinis* subgroup consisting of New World species, and the *obscura* subgroup containing species found in both Old and New Worlds (Fig. 1A). Buzzati-Traverso and Scossiroli (1955) used morphological characters to examine the *affinis* and *obscura* subgroups. They concluded that, within the "traditional" *obscura* subgroup (*sensu* Sturtevant 1942), there was two distinct lineages of Nearctic species and several lineages of Palearctic species. Research conducted in the past 25 years, including allozyme electrophoresis (reviewed in Lakovaara and Saura 1982), mitochondrial restriction site analysis (Latorre, *et al.* 1988; Gonzalez, *et al.* 1990; Barrio, *et al.* 1992), polytene chromosome phylogenies (Brehm, *et al.* 1991), DNA-DNA hybridization (Goddard, *et al.* 1990), and nucleotide sequences (Beckenbach, *et al.* 1993; Barrio, *et al.* 1994; Russo, *et al.* 1995; Barrio and Ayala, 1997), has refined our view of evolution in the *obscura* group. In their review of the *obscura* group phylogeny, Lakovaara and Saura (1982) summarize these data and indicate that the *obscura* subgroup can be divided into

two subgroups, *obscura* and *pseudoobscura* (Fig. 1B). Several phylogenetic analyses have shown the *pseudoobscura* subgroup, which is distributed exclusively in the New World, to be most closely related to species placed in the Nearctic *affinis* subgroup (*e.g.* Lakovaara and Saura 1982; Barrio, *et al.* 1992; Barrio and Ayala 1997). The common ancestor of these species groups likely colonized the western Nearctic region prior to mid-Miocene times, approximately 20 million years ago (Throckmorton 1975). Recent expeditions in sub-Saharan Africa (Tsacas, *et al.* 1985; Cariou, *et al.* 1988) have discovered an additional subgroup, *microlabis*, which is found exclusively in the Afrotropical region (Fig. 1C). Finally, recent nucleotide sequence studies have shown that the *obscura* subgroup (*sensu* Lakovaara and Saura 1982) can be further divided into the *subobscura* and *obscura* subgroups (Fig. 1D; Barrio, *et al.* 1994). The *subobscura* subgroup contains the widespread Palearctic species *D. subobscura* and two island endemics, *D. madeirensis* and *D. guanche*. The revised *obscura* subgroup contains all of the other Palearctic species placed in this group, although, based upon previous studies, it is doubtful that these species form a monophyletic clade (Barrio and Ayala 1997; Gleason, *et al.* 1997).

This study uses individual and combined analyses to examine nucleotide sequences from six mt (Beckenbach, *et al.* 1993; Barrio, *et al.* 1994) and five nu loci (Ruttkay, *et al.* 1992; Russo, et al. 1995; Barrio and Ayala 1997; Grau and Bachman

1997), in an attempt to resolve the uncertain phylogenetic relationships within the *obscura* species group.

Materials and Methods

DNA Sources, Isolation, and Sequencing

Species sampled from the five subgroups currently recognized in the *obscura* species group are shown in Table 1, along with abbreviations used in Figs. 2 and 3. Live *Drosophila* stocks were obtained from the National *Drosophila* Species Resource Center in Bowling Green, Ohio (*D. tolteca* - BG#14012-0201.0 and *D. guanche* - BG#14011-0095.0). Cultures of *D. madeirensis* were obtained from D. Sperlich at the University of Tubingen. Specimens of *D. obscura* were obtained from M. Radak at the University of Belgrade.

Genomic DNA was isolated following the method of Gloor and Engels (1992). Each locus was amplified from the four species in this study using standard PCR cycling conditions. Oligonucleotides 4682 ACAT(TC)CAGCCAIGAGTTGAA(CT)TTGTG, located in the first exon, and 4683 CTGGGIGGCATTGGI(CT)T(CG)GACACCAC, located in the third exon, were used to amplify the alcohol dehydrogenase (*Adh*) gene from *D. tolteca*. PCR products from the *Adh* gene were then cloned into the TA cloning vector (Invitrogen). Several colonies were selected and sequenced using a dsDNA cycle sequencing procedure (GIBCO-BRL) in order to reduce the effects of polymerase error.

Oligonucleotides, designed after Simon, *et al.* (1994), used to amplify the entire 688 bp cytochrome oxidase II (*COII*) gene from *D. guanche*, *D. madeirensis*, and *D. obscura* were (1) ATGGCAGATTAGTGCAATGG and (2) GTTTAAGAGACCAGTACTTG. These products were purified by membrane filtration (Millipore) and sequenced directly using a standard dsDNA cycle sequencing protocol (GIBCO-BRL). Both DNA strands were sequenced from each PCR product. Accession numbers of the sequences generated in this study are listed in Table 2.

Sequence Alignment

Alignment of these sequences was performed by eye and were identical to the published alignment (Ruttkay, *et al.* 1992; Beckenbach, *et al.* 1993; Barrio, *et al.* 1994; Russo, et al. 1995; Barrio and Ayala 1997; Grau and Bachman 1997). The intron regions of *Adh*, *Gpdh*, and *Sod* were excluded from all analyses. The four *Adh* and *COII* sequences determined for this study (described above) were aligned to the literature sequences by eye and required the inclusion of no additional gaps. Accession numbers for all sequences used in this study are shown in Table 2.

Sequence Selection and Taxon Sampling

The following nucleotide partitions were used in individual analyses: *COII*, *cytb*, *ND1*, *ND5*, *16S*, *COI*, *Adh*, *Sod*, *Gpdh*, and *28S* (Figs. 2 and 3). Sequences selected for

individual analyses met the following criteria: (1) at least one nucleotide sequence from four of the five currently recognized *obscura* subgroups was determined and (2) an outgroup sequence was present.

Combined analyses #1 (Figs. 4A and 4B) and #2 (Fig. 5) included all of the DNA sequences included in the individual analyses (above) and the *5S* locus. Nucleotide sequences were included in these analyses if they sampled a representative taxon from at least three of the five obscura species subgroups. It was not necessary for an outgroup taxon to be present. A subset of 16 taxa were examined in combined analyses #1 and #2. Only those taxa which had one sequence determined from both a mitochondrial and nuclear partition were included in these combined analyses.

Phylogenetic Analysis - Individual Loci

To estimate the phylogeny of the *obscura* species group, all loci were analyzed using a variety of tree-building methods, including maximum likelihood (ML), neighbor joining (NJ) and maximum parsimony (MP). We present the ML and MP analyses for the individual analyses (Figs. 2 and 3). Individual MP searches were performed with the branch and bound algorithm (PAUP 4.0; Swofford 1997). The level of support at each node of all most parsimonious trees obtained was assessed using decay indices (DI; Bremer 1988) and bootstrap proportions (BP; Felsenstein 1985; 1988). Five hundred replicates were performed in each bootstrap search. Individual ML analyses employed

the following simple model of evolution: (1) nucleotide frequencies were determined empirically from the data, (2) all sites were assumed to evolve at the same rate, and (3) the transition/transversion ratio was set to 2 (Hasegawa, *et al.* 1985). Support for each clade in the ML trees was determined using bootstrap proportions (Felsenstein 1985; 1988). One hundred replicates were performed for each locus. All trees were rooted using a member of the *melanogaster* species group, either *D. melanogaster* or *D. yakuba*. Since the ML and MP tree topologies were identical, only the MP trees are presented along with likelihood scores from ML analyses.

Partition Homogeneity Test

The partition homogeneity test (PHT), as implemented in PAUP 4.0d59-61 (Swofford 1998) was used to test for incongruence between data sets. The null hypothesis of the PHT is that each pair of loci are as congruent as two randomly generated partitions of equal size. The test compares the length of the most parsimonious tree(s) for the original pair of partitions with a number of randomly generated data sets. The character columns in the random data sets are rearranged, but size of each partition is kept constant. One hundred randomly generated data sets were used to create a null distribution to test the statistical significance of tree lengths from the original partitions. We performed pairwise tests to look for incongruence between individual partitions (Table 2). When comparing individual loci with the PHT, data sets were trimmed to

contain only those taxa in common between the two partitions. We also performed a PHT on the seven taxa that were in common to both the combined nu and combined mt data sets to determine if these partitions were incongruent (Table 3).

Phylogenetic Analysis - Combined Analyses

Only MP was used to analyze combined data partitions. Searches were performed with the branch and bound algorithm (PAUP 4.0d59-61; Swofford 1998 and support for each node was determined as described above. We also used partitioned Bremer support (Baker and DeSalle 1997) to measure the amount of support provided by each individual partition to the DI for every node in the combined analysis phylogenies (Figs. 4 and 5). Partitioned Bremer support (PBS) shows the contribution of each partition to the decay index of every node on the total evidence tree. To obtain the PBS value for a given node on the total evidence tree, the length of the partition on the unconstrained total evidence tree was subtracted from the length of a partition on a tree constrained to not contain the node of interest. If the partition supports a relationship represented by a node in the total evidence tree, then the PBS value will be positive. If, on the other hand, a partition supports an alternative relationship, the PBS value will be negative, indicating incongruence with the simultaneous analysis. The magnitudes of PBS values indicates the level of support for, or incongruence with, a node. The sum of all partition lengths for any given node will always equal the decay index for that node on the

total evidence tree. Using this method allows us to determine the relative contribution of each partition to the different simultaneous analysis trees (Figs. 4 and 5).

Results

Individual Analyses of Nucleotide Data

Figures 2 and 3 show the majority rule bootstrap consensus trees resulting from unweighted analysis of individual mt and nu partitions, respectively. Bootstrap proportions are displayed above and decay indices are shown below each node supported. Some important aspects of each analysis including (1) the total number of characters and total number of parsimony informative characters in each locus, (2) the number and length of most parsimonious trees (MPTs) recovered by each search, (3) the consistency and retention indices for each MPT, and (4) the likelihood score for each tree found in ML searches are shown in each (Figs. 2-5).

The mt *COII* partition (Fig. 2A) gives high support for the traditional phylogeny within the *pseudoobscura*, *obscura*, and *subobscura* subgroups (Throckmorton 1975; Lakovaara and Saura 1982; Beckenbach, *et al.* 1993). This analysis of the *COII* locus shows that the *affinis* and *pseudoobscura* subgroups are sister taxa, although this relationship is not highly supported (BP = 58; DI = 1). Within the *affinis* subgroup, phylogenetic relationships are mostly unresolved. This partition is also unable to determine which subgroup, either the *obscura* or *subobscura*, is the sister group to the

affinis-pseudoobscura clade. Furthermore, this partition indicates that the *obscura* subgroup may not be monophyletic.

The mt cytochrome b partition (*cytb*), determined by Barrio, *et al.* (1994) recovers several monophyletic groups, including the *affinis*, *pseudoobscura*, *obscura*, and *subobscura* subgroups (Fig. 2B). The *cytb* analysis suggests that the *obscura* subgroup may not be monophyletic. This locus is also unable to determine the sister group relationships among any of the subgroups.

The mt *16S* partition (Barrio, *et al.* 1994) is able to resolve the *obscura* and *subobscura* subgroups with high bootstrap support (BP = 81 and 79, respectively), although relationships within these clades are not well resolved (Fig. 2C). This locus also places *D. miranda* within the *affinis* subgroup, indicating that the *affinis* and *pseudoobscura* subgroups are paraphyletic with respect to one another. This result may be due to the relatively few phylogenetically informative characters, 11, in this partition.

Figure 2D shows the phylogenetic relationships inferred from the mt *ND5* partition (Barrio, *et al.* 1994). Although this partition is unable to resolve any relationships among the subgroups, the relationships within each major clade (the *affinis*, *pseudoobscura*, and *obscura* subgroups) are well resolved and highly supported.

The mt *ND1* partition (Barrio, *et al.* 1994) indicates that the *subobscura* and *pseudoobscura* subgroups are monophyletic (Fig. 2E). Although relationships within the *subobscura* subgroup are completely unresolved, those within the *pseudoobscura*

subgroup are congruent with previous work. Interestingly, this partition gives relatively low support (BP = 69; DI = 2) for the sister group relationship between the *affinis* and *subobscura* subgroups, a result that has not been seen before and is in conflict with all other data gathered to date.

The mt *COI* partition (Gleason, *et al.* 1997) indicates that the *affinis*, *pseudoobscura*, and *subobscura* subgroups are monophyletic (Fig. 2F). The *obscura* subgroup comes out in two non-monophyletic clades. This locus, however, is unable to recover any information regarding the relationships among the subgroups examined.

Figure 3A shows the phylogeny obtained from parsimony analysis of the nu *Sod* partition (Barrio and Ayala 1997). In this tree the *affinis* and *pseudoobscura* subgroups are fully resolved and placed as sister taxa with high support (BP = 100; DI = 7). The *subobscura* subgroup, which is also highly supported (BP = 96; DI = 5), is shown, along with part of the *obscura* subgroup, to be the sister taxa of the *affinis-pseudoobscura* clade (Fig. 3A).

The nu *Gpdh* gene tree is shown in Fig. 3B (Barrio and Ayala 1997). Though the obscura subgroup itself is not monophyletic, the *obscura* and *subobscura* subgroups together form a monophyletic clade (BP = 95; DI = 6). The *pseudoobscura* subgroup is a sister taxon to the *obscura-subobscura* clade (BP = 95; DI = 8). This result is similar to the traditional "two subgroup" view of Sturtevant (1942). Finally, the *affinis* subgroup is not monophyletic and is a sister taxon to the other subgroups.

The tree for the nu *Adh* locus is shown in Fig. 3C. Although sequences from fewer taxa than in the other individual studies have been determined, there is at least one representative from 4 of the 5 *obscura* subgroups. This partition gives results similar to the other partitions in this study. The *pseudoobscura* and *subobscura* subgroups are shown to be monophyletic (BP = 100). There is no resolution of among-subgroup relationships in this phylogeny. However, there is strong support for some within-subgroup relationships, such as within the *subobscura* and *pseudoobscura* subgroups.

The results of the individual analysis of the *28S* (Ruttkay, *et al.* 1992) locus is shown in Fig. 3D. This locus is largely unresolved with respect to relationships within and among the five subgroups of the *obscura* species group. Only the *microlabis* subgroup is shown to be monophyletic; the *pseudoobscura*, *subobscura*, *affinis* and *obscura* subgroups are all non-monophyletic.

Summary of Individual Analyses

The *COI*, *COII*, *cytb*, *ND5*, and *Sod* loci indicate that the *affinis* subgroup is monophyletic. The *16S*, *28S*, *ND1* and *Gpdh* loci are unable to resolve the monophyly of the *affinis* subgroup clade. With the exception of the *16S* and *28S* loci, all individually analyzed sequences indicate that the *pseudoobscura* subgroup is monophyletic. The Nearctic species (the *affinis* and *pseudoobscura* subgroups) are monophyletic in the mt *COII* and nu *Sod* analyses. The mt *ND1* analysis places the *affinis* subgroup as a sister

clade to the *subobscura* subgroup species and the nu *Gpdh* gene suggests that the *pseudoobscura* subgroup forms a monophyletic clade with the Old World species. The *28S* analysis proposes that part of the *obscura*, *subobscura* and *pseudoobscura* subgroups form a clade, a finding that is not seen in any other analysis performed. All other loci analyzed individually are unable to determine relationships among any of the *obscura* subgroups. With the exception of the *28S* locus, the *subobscura* subgroup is monophyletic in all individual analyses, although sister group relationships within this subgroup sometimes differ. Within the *obscura* subgroup, *D. bifasciata* and *D. subsilvestris* are difficult to place. With the exception of the *16S* locus, which places these species in the *obscura* subgroup, and the *Gpdh* locus, which implies that these species form a sister clade to the *subobscura* subgroup, the relationships of these species are unresolved.

Comparisons of Individual Partitions

The PHT, as implemented in PAUP* 4.0 (Swofford 1998), is employed to make pairwise comparisons between each individually analyzed partition in this study. The mitochondrial partitions are, for the most part, congruent with one another. The PHT indicates three points of incongruence within the six mt data sets: *ND1* and *COI*

partitions are both incongruent with the *COII* partition and the *COI* partition is incongruent with the *cytb* locus (Table 3). The four nu loci compared are incongruent in four comparisons (Table 3). The *Sod* gene is incongruent with all other nu partitions and the *Gpdh* gene is incongruent with *28S*. In comparisons between nu and mt loci, the nu *Adh* and *28S* loci are the most congruent loci, each only being incongruent with one mt locus (*COI* and *16S* respectively; Table 3). The nu *Sod* gene is incongruent with all mt partitions except for *COII* (Table 3). The nu *Gpdh* partition is congruent with only the mt *COII* and *cytb* genes (Table 3). These results indicate that the degree of incongruence among the loci in this study falls on a continuum, with some partitions being more incongruent than others.

Combined Analysis #1 - Nuclear *vs.* Mitochondrial Partitions

The individual data partitions were then combined into two data sets, nu and mt, to determine if different classes of data (*i.e.*, non-recombining, maternally inherited *vs.* recombining, bi-parentally inherited) yield different topologies when analyzed (Figs. 4A and 4B) or are significantly incongruent with one another when compared with the PHT test (Table 2). Figure 4A shows the phylogeny derived from simultaneous analysis of all mt data sets. The *affinis*, *pseudoobscura*, *obscura*, and *subobscura* subgroups are each resolved, well supported, and monophyletic (BP = 85; DI = 3, BP = 100; DI = 19, and BP = 76; DI = 5, respectively). These data are, however, unable to completely resolve

relationships among any of the subgroups. Figure 4B presents the phylogeny derived from the combined analysis of all nu partitions. This tree also shows the *affinis*, *pseudoobscura*, and *subobscura* subgroups to be resolved, well supported, and monophyletic (BP = 96; DI = 5, BP = 100; DI = 16, and BP = 78; DI = 4, respectively). This partition shows that species from the *subobscura* and *obscura* species subgroups form a weakly supported monophyletic group (BP = 55; DI = 4), although the exact phylogenetic relationships within this clade are unresolved (Fig. 4B). The *pseudoobscura* subgroup, in conflict with some previous data, is shown to be the sister taxon of the *subobscura-obscura* clade, however support for this relationship is weak (BP = 52, DI = 4). Other topological differences between trees are either due to a lack of resolution or the shifting of relationships between species within the same subgroup. A partition homogeneity test indicates that these data partitions are not significantly heterogeneous with respect to one another (Table 3).

PBS values (Baker and DeSalle 1997) are used here to determine which partitions were lending support to any given node on the trees from the two combined analyses (Figs. 4A and 4B). Excepting those differences due to lack of resolution, the MPTs from the two combined analyses (Figs. 4A and 4B) differ primarily in the relationships among *D. ambigua*, *D. tristis*, and *D. obscura* within the *obscura* subgroup. Support for the ((*D. ambigua*, *D. tristis*), *D. obscura*) relationship seen in the combined nu analysis

comes only from the *5S* (PBS = 2). Support for the ((*D. ambigua*, *D. obscura*), *D. tristis*) is given by *COI*, *ND1* and *ND5* (PBS = 14).

The PBS values for each locus can be summed across all nodes on the MPTs from combined analyses to give a notion about how much any one partition is contributing to the entire combined analysis topology. *COII*, *COI*, *ND1*, and *16S* have positive PBS sums (58, 31.7, 27.3, and 0.7, respectively), *cytb* has a negative PBS sum (-8.7), and the PBS values for *ND5* sum to zero. PBS sums for the combined nu analyses show that the *5S*, *Gpdh*, and *Adh* loci give positive values (47.85, 29.3, and 9.8, respectively) and *Sod* and *28S* give negative values (-3.95 and -4, respectively). The high value for the *5S* locus is somewhat misleading, however, as it is due primarily to two nodes (PBS = 17.5 and 26). It should also be noted that, even though a locus may have a high positive sum of PBS values, it does not necessarily lend support at all nodes. The reverse is also true. For example, although the *Sod* gene has an negative summed PBS (-3.95), it is congruent with (*i.e.* has either a positive or 0 PBS value) 7 of the 12 nodes in the combined analysis phylogeny.

Combined Analysis #2 - Nuclear and Mitochondrial Data

The combined nu and combined mt data sets were then combined to determine if simultaneous analysis of mt and nu partitions could resolve the phylogeny of the *obscura* species group better than the combined analysis of those two partitions alone (Figs. 4A

and B). Figure 5 shows the phylogeny which results from the combined analysis of the nu and mt partitions. The *affinis*, *pseudoobscura*, *obscura* and *subobscura* subgroups all form monophyletic clades. Notably, the *subobscura* and *obscura* subgroups form a monophyletic clade which is fairly well supported (BP = 77, DI = 7). Even though this relationship was not seen in the combined nuclear analysis (Fig. 4B), the nu *5S* locus contributes positively to the decay index at this node (PBS = 2). The conflict between the combined nu and combined mt analyses in estimating relationships within the *obscura* subgroup, (AMB,OBS),TRI)) in the mt phylogeny *vs.* (TRI,AMB),OBS)) in the nu phylogeny, is resolved and identical to the one supported by the mt partition. This combined analysis is, however, unable to resolve the relationships among the *obscura-subobscura*, *pseudoobscura* and *affinis* clades.

Discussion

Combining and Partitioning Data in Phylogenetic Analysis

One of the major debates in systematics is whether data obtained from independent sources (i.e., morphology vs. molecular data) should be analyzed separately or in a combined analysis (de Queiroz, *et al.* 1995; Brower, *et al.* 1996). This controversy will continue as more independent data sets are gathered and brought to bear on phylogenetic questions. Some support the notion that simultaneous analysis maximizes explanatory power and is, therefore, superior (Kluge 1989; Brower, *et al.* 1996), while

others believe that it is preferable to present a consensus of individual analyses (Miyamoto and Fitch 1995). A compromise position, referred to as conditional combination, proposes that data partitions be subjected to a test of congruence and then, if congruent, combined (Bull, et al 1993; de Queiroz, *et al.* 1995). Data sets that are incongruent with other partitions are excluded from simultaneous analysis. One could employ either strict or permissive criteria when deciding which data partitions to include or exclude in a phylogenetic analysis. Under a strict criterion, only those partitions which were congruent with all partitions in the study are included. The permissive approach would include all partitions that are at least partially congruent with the other partitions, only those partitions which are incongruent with all other partitions are excluded.

The nucleotide sequence data for the *Drosophila obscura* group offers a unique opportunity to examine the conditional combination approach to phylogenetic analysis. The PHT indicates that the nu *Sod* partition is congruent with the mt *COII* partition, even though *Sod* is incongruent with all of the other loci examined in this study and *COII* is congruent with all loci except for *ND1* (Table 3). Examining the *Sod* phylogeny indicates that, while it does differ in placement of some groups, it is in agreement with some other relationships proposed by each individual gene tree (Figs. 2 and 3). It may be the case that incongruence between data partitions, as suggested by a significant PHT, is seldom complete. The PBS values that we have presented for each node in our combined analyses seem to bear this out. With the exception of *28S* (PBS = 0), all loci contribute

positive values at some nodes in combined analysis #2. Furthermore, many of those loci which have high positive summed PBS values also conflict (*i.e.*, have negative PBS values) at some nodes. It may, in fact, be more accurate to view congruence and incongruence between partitions as a continuum, rather than as an absolute characteristic of a set of partitions. Some data sets may effectively track historical relationships at one taxonomic level, while contributing to incongruence at another.

We feel that it is most appropriate to combine all the data in a simultaneous analysis, even though some partitions are incongruent with one another when compared with a PHT, because (1) the strict combination criterion can result in the omission of large amounts of data, some of which may be largely congruent with the included data, making it difficult to determine which partitions (or parts of partitions) to exclude from the analysis and (2) examination of PBS values for each combined analysis (Figs. 4-5), indicates that each partition does have some degree of influence, either positive or negative, on the topology of simultaneous analysis trees.

Phylogeny of the *Drosophila obscura* species group

The phylogeny of the *obscura* species group has been a matter of debate almost since the group was first proposed (Sturtevant 1942; Buzzati-Traverso and Scossiroli (1955). Several different types of data, including morphological, polytene chromosome banding patterns, allozymes, restriction fragment length polymorphisms, DNA-DNA

hybridization, 1- and 2-D gel electrophoresis, and nucleotide sequences have gradually refined our view of evolution in this group. Phylogenetic analyses indicate that individual data sets (Figs. 2 and 3), as well as some combined data sets (Figs. 4A and 4B), are unable to resolve the sister group relationships between the *affinis*, *obscura*, *subobscura*, *pseudoobscura* and *microlabis* subgroups. Combined analysis of the 11 nu and mt nucleotide sequences in this study (Fig. 5) does indicate that the *subobscura* and *obscura* subgroups are sister taxa. It is clear that, even with large numbers of characters (over 6300, in combined analysis #2), resolving the relationships among the major lineages within the *obscura* species group is not a trivial problem, perhaps because of the rapid manner that this group is thought to have formed (Throckmorton 1975; Gleason, *et al.* 1997).

Figure 6 presents two hypotheses of phylogenetic relationships within the *obscura* species group and serves to summarize our current understanding of evolution in this species group. Figure 6A shows a possible hypothesis of evolutionary relationships within the *obscura* group, along with the cladistic analyses from the present study which support each node. Figure 6B is the *obscura* group phylogeny based on mt DNA sequences presented by Gleason, *et al.* (1997). Both studies suggest that there are two major clades in the *obscura* group, the Old World *obscura* and *subobscura* clade and the New World *affinis* and *pseudoobscura* clade. They differ only in the placement of the

microlabis subgroup which may be either a close relative of the *subobscura* subgroup (Fig. 6A) or the sister taxon to the remaining *obscura* species (Fig. 6B).

The present study proposes that the Afrotropical *microlabis* subgroup forms a clade with the *subobscura* subgroup, which has a distribution which includes parts of northern Africa. This *microlabis-subobscura* clade is nested within a larger Old World clade which contains the remaining species placed in the *obscura* subgroup. Support for this hypothesis comes from biogeographic information (Throckmorton 1975), previous morphological and molecular studies (Lakovaara and Saura 1982; Cariou, *et al.* 1988; Ruttkay, *et al.* 1992), and cladistic analyses (Fig. 6 and data not shown). The proposed *subobscura-microlabis* ancestor may have colonized high elevation habitats in the Afrotropical region and speciated there. A similar pattern of colonization of high elevation habitats in tropical regions is also observed in members of the *affinis* and *pseudoobscura* subgroups in the New World tropics (Throckmorton 1975; Heed and O'Grady, submitted). It is possible that these species are ecologically better adapted to the "temperate-like" habitats found at high elevations in the tropics and are able to diversify there via chance colonization events (Throckmorton 1975).

Alternatively, Gleason, *et al.* (1997) propose that the *microlabis* subgroup is basal within the *obscura* species group and diverged soon after these species split from the *protomelanogaster* ancestor (Fig. 6B). They state that the species in the *microlabis* subgroup "are early relicts of this split and therefore have the deepest branches in the

phylogeny (Gleason, *et al.* 1997; p. 437)." This is in conflict with Throckmorton's (1975) biogeographic studies, which suggests that "although several species have distributions bordering or extending into the tropics, there is no indication that any of these represents ancestral links... (p. 437)." Furthermore, a "basal" *microlabis* subgroup is also incongruent with some of the studies Gleason, *et al.* (1997) cite as supporting this relationship (see Cariou, *et al.* 1988; Ruttkay, *et al.* 1992).

The hypotheses proposed in Fig. 6 provide a tentative view of evolution in the *obscura* species group and should be tested with additional data in the future. Representation of species from the *microlabis* subgroup, which have been absent in many previous molecular studies, in future studies is clearly required. Combined analyses of diverse data may provide some hints concerning the relationships within the *obscura* species group, but many more characters may be required before relationships within this phylogenetically complex taxon can be fully resolved.

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Table 1

Taxonomy of the *Drosophila obscura* species group with abbreviations used in this study.

affinis subgroup

D. affinis - AFF

D. algonquin - ALG

D. athabasca - ATH

D. azteca - AZT

D. narragansett - NAR

D. tolteca - TOL

microlabis subgroup

D. kitumensis - KIT

D. microlabis - MIC

subobscura subgroup

D. guanche - GUA

D. madeirensis - MAD

D. subobscura - SOB

pseudoobscura subgroup

D. lowei - LOW

D. miranda - MIR

D. persimilis - PER

D. pseudoobscura - PSE

D. pseudoobscura "bogotana" - PBO

obscura subgroup

D. ambigua - AMB

D. bifasciata - BIF

D. imaii - IMA

D. obscura - OBS

D. subsilvestris - SSI

D. tristis - TRI

Table 2

Genbank accession numbers of the *Drosophila obscura* group sequences used in this study

	COI	*COII*	*cytb*	*ND5*	*16S*	*ND1*	*Sod*	*Gpd*	*Adh*	*5S*	*28S*
AFF	U51604	M95140	U07273	U07272	U07277	U07274	U47879	U47874	-	-	X71207
ALG	-	M95144	U07279	U07278	U07281	U07280	-	-	-	-	-
ATH	-	M95141	-	-	-	-	-	-	-	-	-
AZT	U51605	M95146	U07283	U07282	U07285	U07284	U47866	U47875	-	-	X71205
NAR	-	M95149	-	-	-	-	-	-	-	-	-
TOL	-	M95152	-	-	-	-	U47867	U47876	XXXX	-	-
KIT	-	-	-	-	-	-	-	-	-	-	1
MIC	-	-	-	-	-	-	-	-	-	-	1
LOW	-	M95142	-	-	-	-	-	-	-	-	-
MIR	U51608	M95148	U07317	U07316	U07319	U07318	U47870	U47882	M60998	-	-
PER	U51609	M95143	U07327	U07324	U07329	U07328	U47873	U47886	M60997	-	1
PSE	U51606	M95145					U47871	U47885	X68164	U58691	X71203
PBO	U51607	-	U07321	U07320	U07323	U07322	U47872	U47891	M60994	-	-

Table 2

Genbank accession numbers of the *Drosophila obscura* group sequences used in this study (continued)

AMB	U51610	M95150	U07297	U07296	U07299	U07298	U47868	U47880	X54813	U58687	1
BIF	U51611	M95147	U07313	U07312	U07315	U07314	U47869	U47883	U40986	U58694	1
GUA	U51612	XXXX	U07326	U07294	U07295	U07294	U47889	U47878	X60113	U58703	1
IMA	-	-	-	-	-	-	-	-	U40987	U58692	-
MAD	U51613	XXXX	U07325	U07290	U07292	U07291	U47887	U47890	X60112	U58710	-
OBS	U51614	XXXX	U07301	U07300	U07303	U07302	U47892	U47881	-	U58715	1
SOB	U51615	M95151	U07287	U07286	U07289	U07288	U47888	U47877	M55545	U58721	1
SSI	U51616	-	U07309	U07308	U07311	U07310	-	U47884	-	U58695	-
TRI	U51617	-	U07305	U07304	U07307	U07306	-	-	-	U58707	1
OUT	X03240	X03240	X03240	X03240	X03240	X03240	X13780	X14179	M17833	-	X71167
	U51619	J01404	J01404	J01404	J01404	J01404			X54120		X71159

1. These sequences are not present in GenBank. They were taken directly from Ruttkay, *et al.* (1992).

Table 3

Results of Pairwise Partition Homogeneity Tests

	COII	*ND1*	*ND5*	*cytb*	*16S*	*COI*	*Adh*	*Sod*	*Gpdh*	*28S*	comb.
COII	-	0.05*	0.26	0.55	0.21	0.10*	1.0	0.53	0.23	0.65	
ND1		-	0.51	0.16	0.76	0.28	0.55	0.01*	0.01*	0.68	
ND5			-	0.58	0.60	0.35	1.0	0.02*	0.03*	0.36	
cytb				-	0.56	0.01*	1.0	0.07*	0.23	0.24	
16S					-	0.45	1.0	0.01*	0.04*	0.01*	
COI						-	0.03*	0.01*	0.01*	0.74	
Adh							-	0.10*	1.0	0.44	
Sod								-	0.01*	0.03*	
Gpdh									-	0.05*	
28S										-	mt
comb.										nu	0.16

* Data partitions which display significant homogeneity when compared

Figure Legends

FIG. 1. - Taxonomic history of the five subgroups in the *Drosophila obscura* species group, with references.

FIG. 2. - Results of individual analyses of mt DNA sequences. Each phylogeny is shown with the length, in base pairs (bp) of the partition, the number of parsimony informative (PI) characters in the data set, the number of MPTs found, the number of steps on each MPT, and the ensemble consistency and retention indices (CI and RI, respectively). BPs are shown above and DIs are shown below each node.

FIG. 3. - Results of individual analyses of nu DNA sequences. Each phylogeny is shown with the length, in base pairs of the partition, the number of parsimony informative characters in the data set, the number of MPTs found, the number of steps on each MPT, and the CI and RI values. BPs are shown above and DIs are shown below each node.

FIG. 4A. - Results of combined mitochondrial analysis. Each phylogeny is shown with the length, in base pairs of the partition, the number of parsimony informative characters in the data set, the number of MPTs found, the number of steps on each MPT, and the

CI and RI values. BPs, DIs, and PBS values for each node are indicated. Branch lengths are not proportional to change, they vary only for clarity.

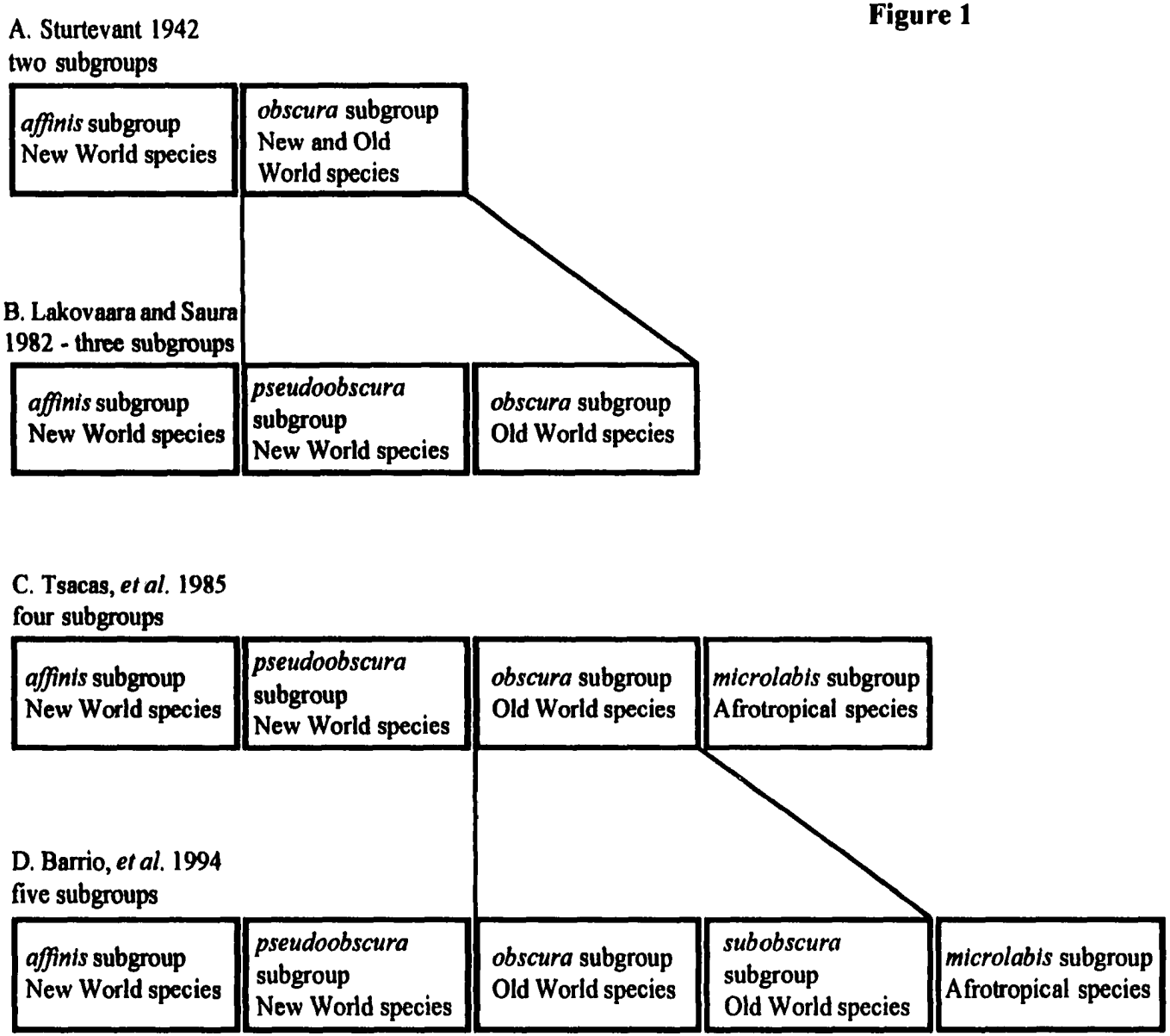
FIG. 4B. - Results of combined nuclear analysis. Each phylogeny is shown with the length, in base pairs of the partition, the number of parsimony informative characters in the data set, the number of MPTs found, the number of steps on each MPT, and the CI and RI values. BPs, DIs, and PBS values for each node are indicated. Branch lengths are not proportional to change, they vary only for clarity.

FIG. 5. - Results of combined analysis #2. Each phylogeny is shown with the length, in base pairs of the partition, the number of parsimony informative characters in the data set, the number of MPTs found, the number of steps on each MPT, and the CI and RI values. BPs, DIs, and PBS values for each node are indicated. Branch lengths are not proportional to change, they vary only for clarity.

FIG. 6. - Summary of phylogenetic relationships within the *Drosophila obscura* species group. (A). Phylogenetic relationships supported by individual and combined analyses in this study. (B). Phylogenetic relationships proposed by Gleason, *et al.* (1997) based on analysis of mtDNA sequences. Figure numbers of the analysis in the present study supporting each node is shown above that node. Numbers associated with named clades

correspond to references which support that clade. 1. Dobzhansky (1935), 2. Sturtevant (1942), 3. Buzzati-Traverso and Scossiroli (1955), 4. Throckmorton (1975), 5. Anderson, *et al.* 1977, 6. Lakovaara and Saura (1982), 7. Cariou, *et al.* 1988, 8. Goddard, *et al.* (1990), 9. Bachmann, *et al.* (1992), 10. Ruttkay, *et al.* (1992), 11. Beckenbach, *et al.* (1993), 12. Bachmann and Sperlich (1993), 13. Krimbas 1993, 14. Barrio, *et al.* 1994, 15. Powell and DeSalle (1995), 16. Russo, *et al.* (1995), 17. Wells (1996), 18. Barrio and Ayala (1997), 19. Gleason, *et al.* (1997), 20. This study.

Figure 1


A. Sturtevant 1942
two subgroups
affinis subgroup
New World species
obscura subgroup
New and Old
World species
B. Lakovaara and Saura
1982 - three subgroups
affinis subgroup
New World species
pseudoobscura
subgroup
New World species
obscura subgroup
Old World species
C. Tsacas, *et al.* 1985
four subgroups
affinis subgroup
New World species
pseudoobscura
subgroup
New World species
obscura subgroup
Old World species
microlabis subgroup
Afrotropical species
D. Barrio, *et al.* 1994
five subgroups
affinis subgroup
New World species
pseudoobscura
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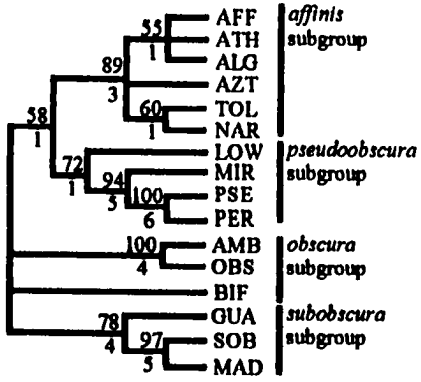
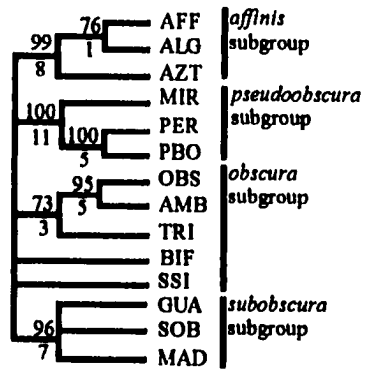
obscura subgroup
Old World species
subobscura
subgroup
Old World species
microlabis subgroup
Afrotropical species

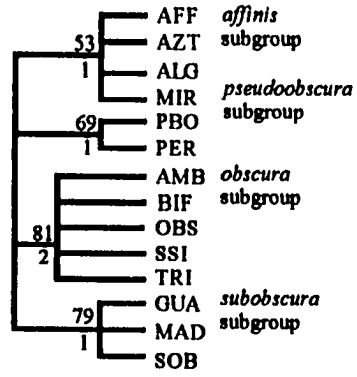
Figure 2



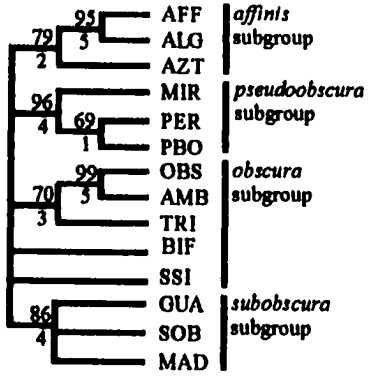
A. COII (688bp, 112 PI)
1 MPT, 361 steps
CI = 0.607, RI = 0.609
-lnL = 2866.1422



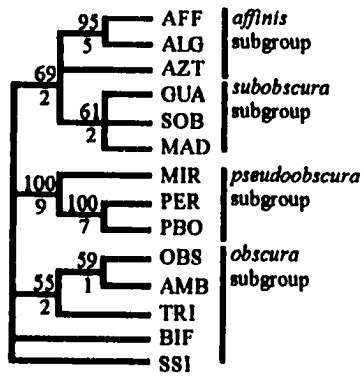
B. CYTB (627bp, 104 PI)
3 MPTs, 319 steps
CI = 0.577, RI = 0.603
-lnL = 2511.7745



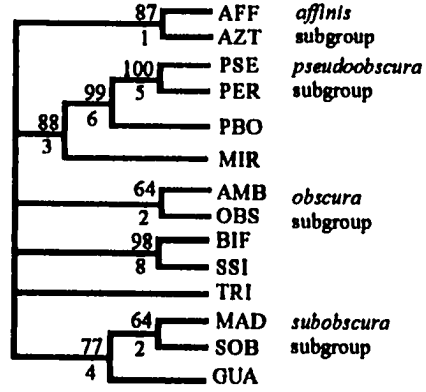
C. 16S (301bp, 11 PI)
19 MPTs, 28 steps
CI = 0.857, RI = 0.889
-lnL = 563.7127



D. ND5 (409bp, 69 PI)
7 MPTs, 231 steps
CI = 0.606, RI = 0.586
-lnL = 1777.6440

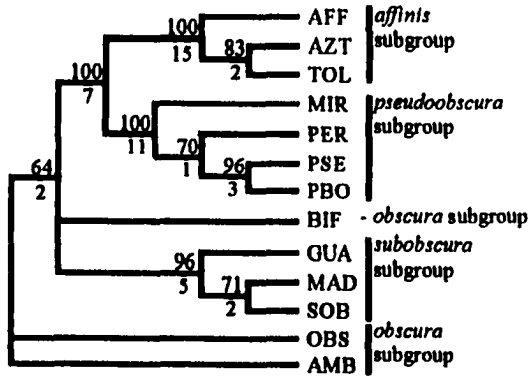


E. ND1 (610bp, 74 PI)
2 MPTs, 216 steps
CI = 0.593, RI = 0.614
-lnL = 2014.6956

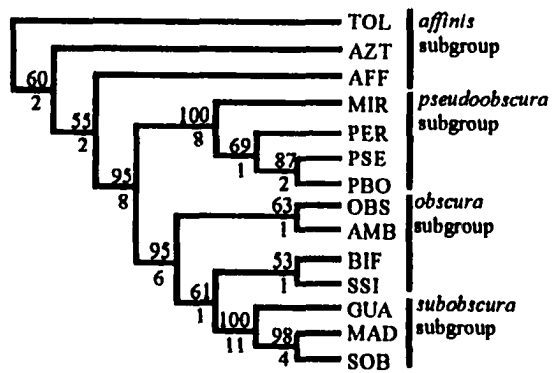


F. COI (496bp, 96 PI)
2 MPTs, 289 steps
CI = 0.592, RI = 0.574
-lnL = 2232.3905

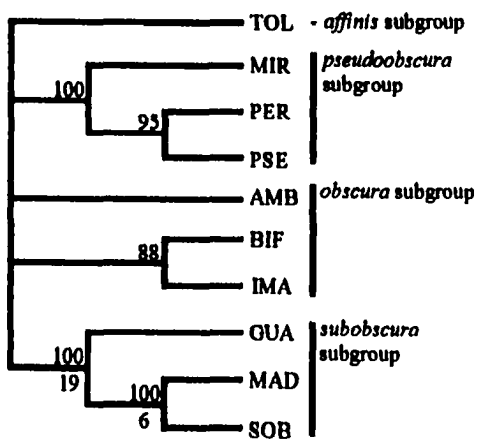
Figure 3



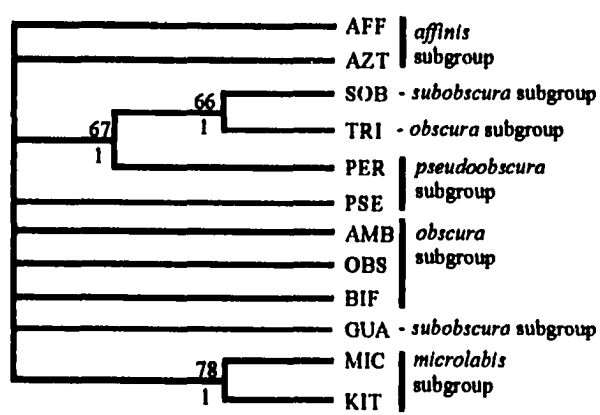
A. SOD (774bp, 134 PI)
2 MPTs, 320 steps
CI = 0.878, RI = 0.894
-lnL = 2772.7103



B. GPDH (759bp, 76 PI)
1 MPTs, 231 steps
CI = 0.823, RI = 0.812
-lnL = 2242.3918

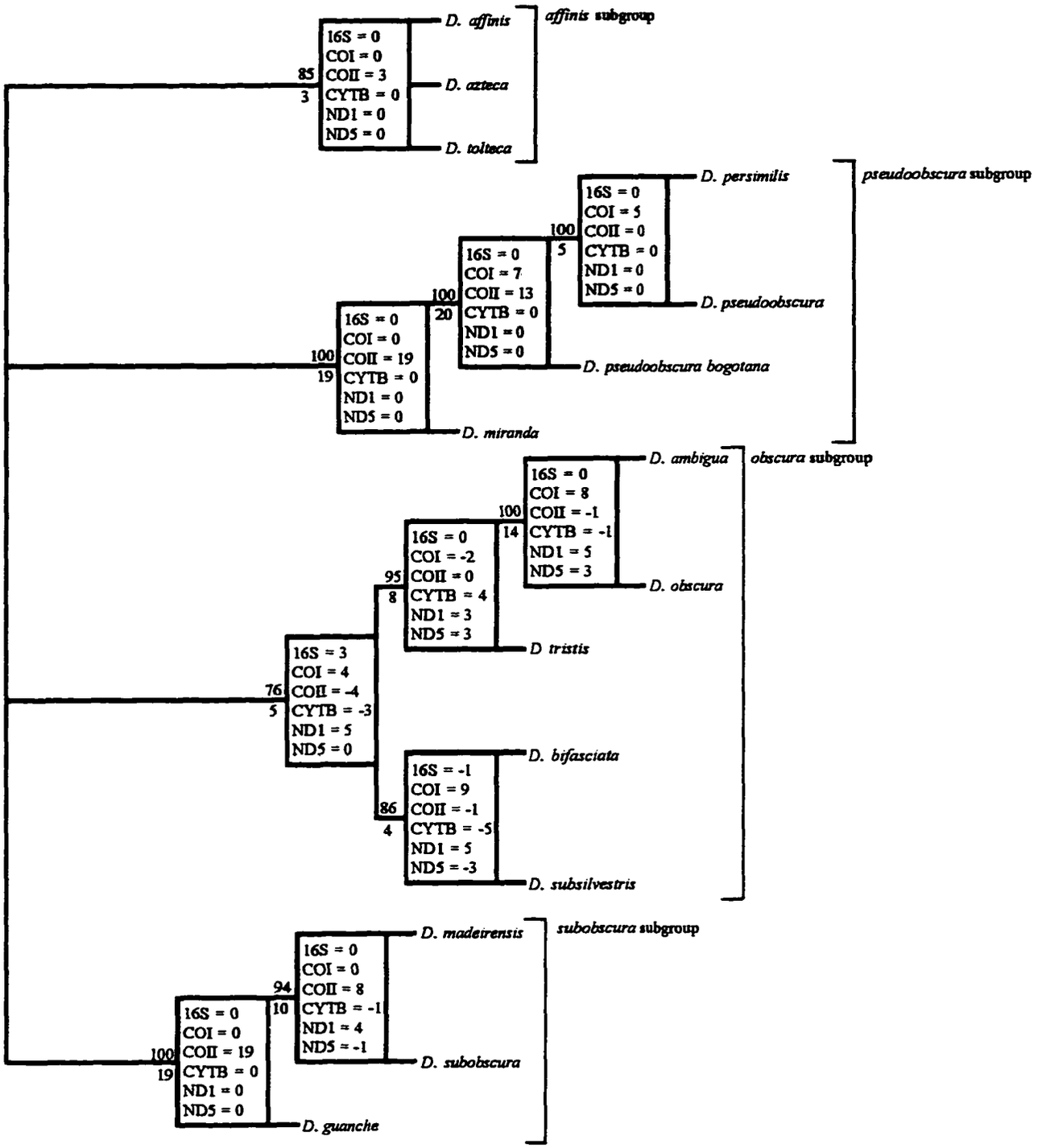


C. ADH (771bp, 84 PI)
2 MPTs, 228 steps
CI = 0.860, RI = 0.815
-lnL = 2337.5312



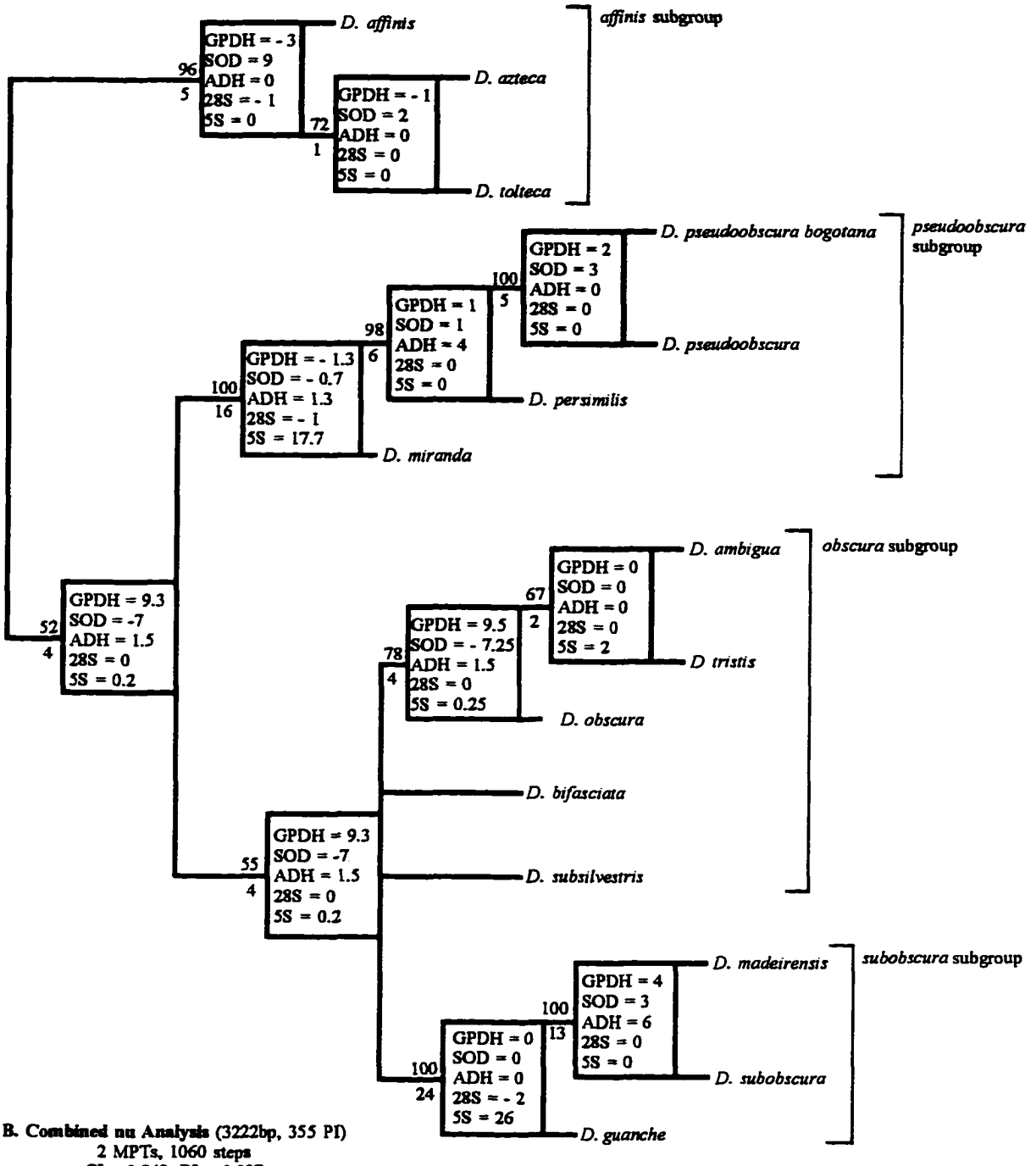
D. 28S (362bp, 5 PI)
6 MPTs, 31 steps
CI = 0.968, RI = 0.889
-lnL = 605.9384

Figure 4A



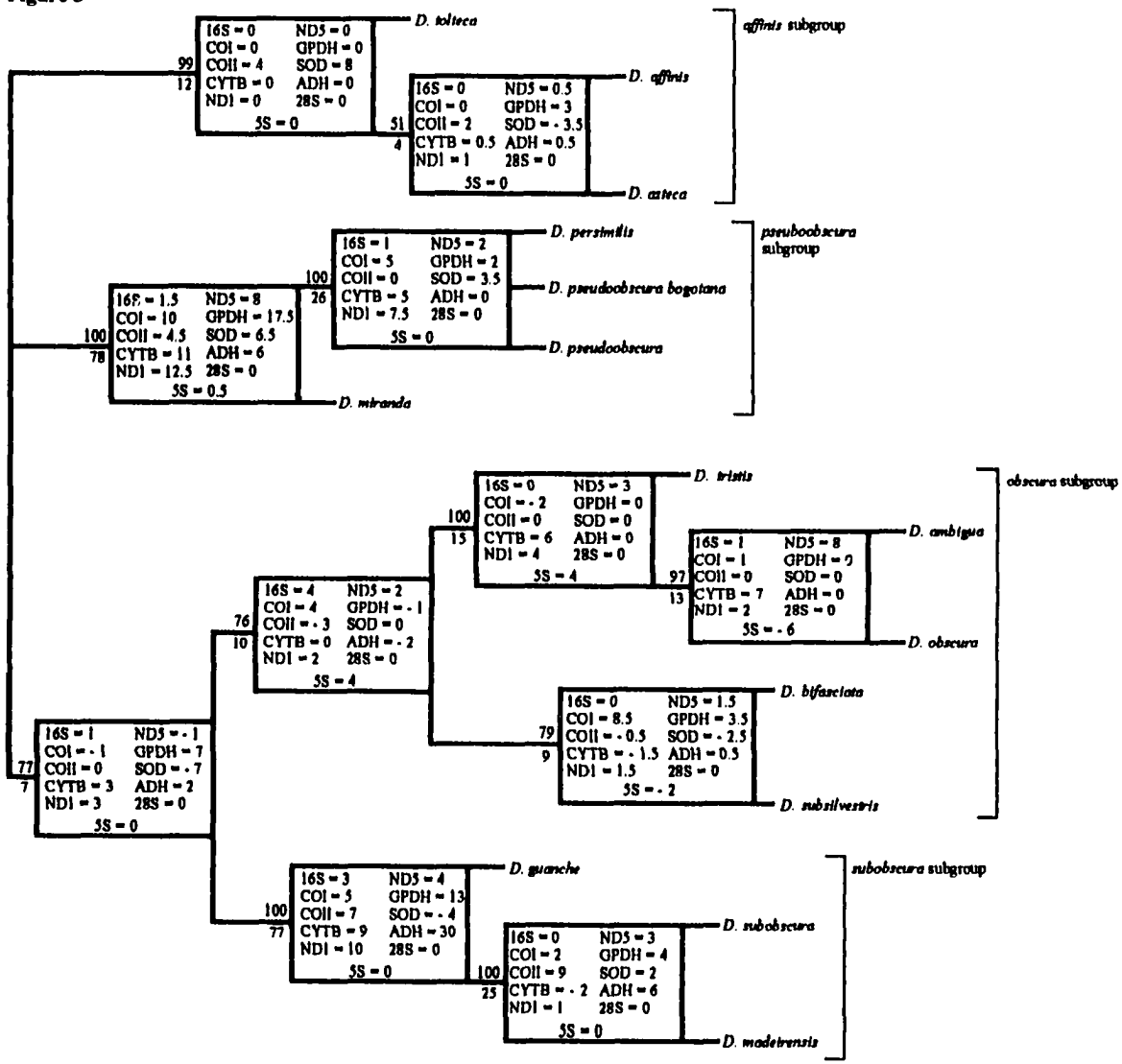
A. Combined mt Analysis (3131bp, 458 PI)
2 MPTs, 1438 steps
CI = 0.596, RI = 0.562

Figure 4B



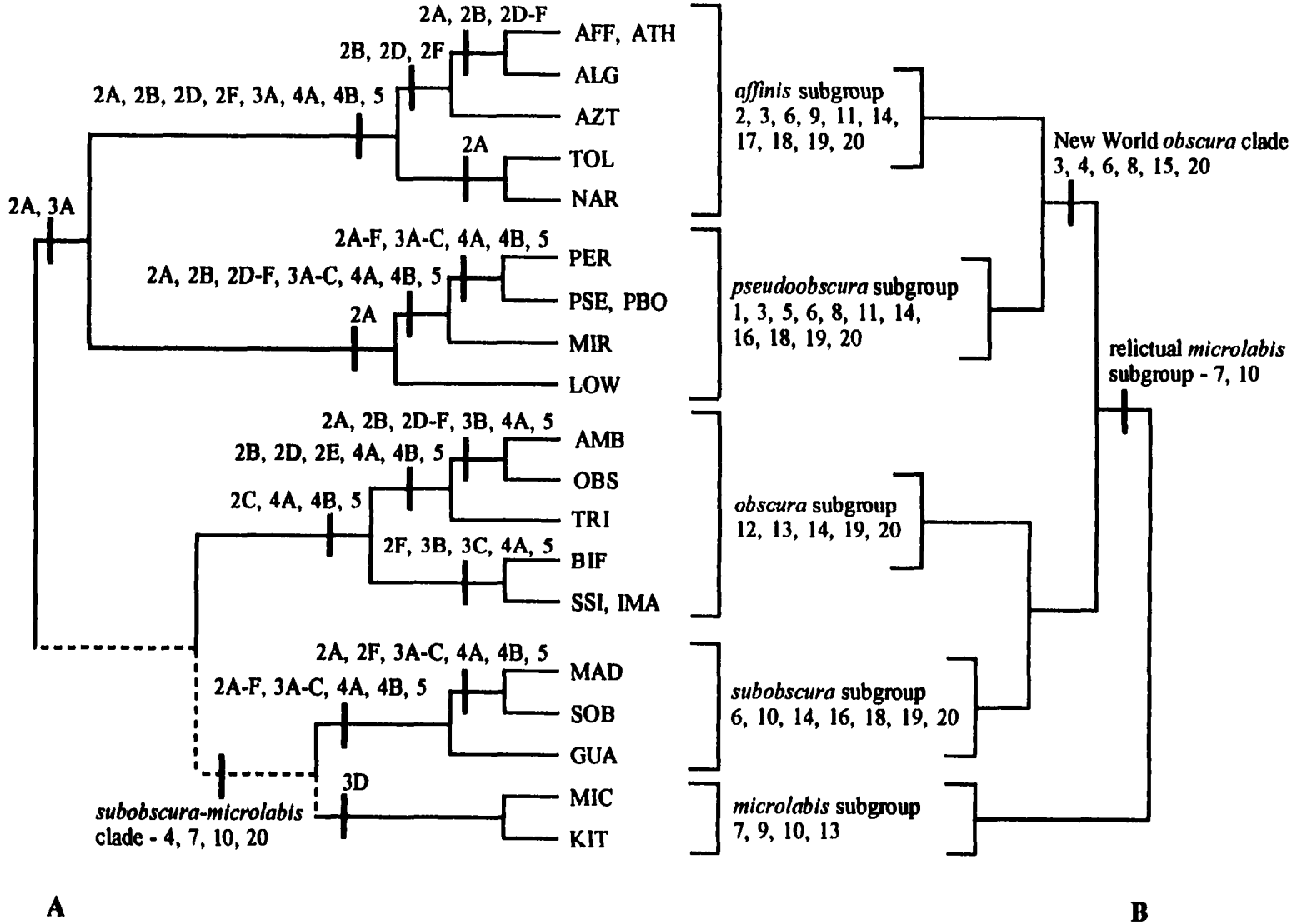
B. Combined nu Analysis (3222bp, 355 PI)
2 MPTs, 1060 steps
CI = 0.842, RI = 0.807

Figure 5



Combined mt and nu Analysis (6353bp, 813 PI)
2 MPTs, 2515 steps
CI = 0.696, RI = 0.651

Figure 6


2A, 2B, 2D-F
AFF, ATH
2B, 2D, 2F
ALG
2A, 2B, 2D, 2F, 3A, 4A, 4B, 5
AZT
2A
TOL
NAR
2A, 3A
2A-F, 3A-C, 4A, 4B, 5
PER
2A, 2B, 2D-F, 3A-C, 4A, 4B, 5
PSE, PBO
2A
MIR
LOW
2A, 2B, 2D-F, 3B, 4A, 5
AMB
2B, 2D, 2E, 4A, 4B, 5
OBS
2C, 4A, 4B, 5
TRI
2F, 3B, 3C, 4A, 5
BIF
SSI, IMA
2A, 2F, 3A-C, 4A, 4B, 5
MAD
2A-F, 3A-C, 4A, 4B, 5
SOB
GUA
3D
MIC
subobscura-microlabis
clade - 4, 7, 10, 20
KIT
affinis subgroup
2, 3, 6, 9, 11, 14,
17, 18, 19, 20
pseudoobscura subgroup
1, 3, 5, 6, 8, 11, 14,
16, 18, 19, 20
obscura subgroup
12, 13, 14, 19, 20
subobscura subgroup
6, 10, 14, 16, 18, 19, 20
microlabis subgroup
7, 9, 10, 13
New World obscura clade
3, 4, 6, 8, 15, 20
relictual microlabis
subgroup - 7, 10
A
B

APPENDIX C

PHYLOGENY OF THE SUBGENUS *SOPHOPHORA* (DIPTERA: DROSOPHILIDAE) INFERRED USING THE 28S, ALCOHOL DEHYDROGENASE, AND CYTOCHROME OXIDASE II GENES

Phylogeny of the subgenus *Sophophora* (Diptera:Drosophilidae) based on combined analysis of nuclear and mitochondrial sequences

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running head: *Sophophora* phylogeny

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Abstract

Sequences from the nuclear (nu) alcohol dehydrogenase (*Adh*) gene, the nu *28S* r (ribosomal) RNA locus and the mitochondrial (mt) cytochrome oxidase II (*COII*) gene were used both individually and in several combined analyses to infer the phylogeny of the subgenus *Sophophora* (Diptera:Drosophilidae:*Drosophila*). Our data suggest that the *melanogaster* and *obscura* species groups form a clade, even though the *melanogaster* group itself may not be monophyletic. Furthermore, the Neotropical *saltans* and *willistoni* species groups also form a monophyletic assemblage, as previous studies have suggested (Pelendakis, et al. 1991; Pelendakis and Solignac 1993). Within the Neotropical clade, the *saltans* species group is strongly supported as monophyletic. Several analyses indicate that the *willistoni* species group is paraphyletic with respect to the *saltans* species group. Finally, use of a larger data set, both in terms of numbers of characters and numbers of taxa, suggests that the subgenus *Sophophora* is monophyletic, contrary to the previously published individual analysis of *28S* rRNA sequences (Pelendakis, et al. 1991; Pelendakis and Solignac 1993).

Introduction

The subgenus *Sophophora* contains several species, including *Drosophila melanogaster* and *Drosophila pseudoobscura*, which have served as important model systems in the study of genetics, ecology, behavior, evolution, and developmental biology. The group was ereced by Sturtevant (1939; 1942) when he subdivided the genus *Drosophila* into subgenera and species groups. The subgenus *Sophophora* was described based on the type species *Drosophila melanogaster* and originally included four species groups, *melanogaster*, *obscura*, *saltans* and *willistoni* (Sturtevant 1939; 1942). Three additional subgroups, *dispar*, *fima* and *dentissima*, were added as more was learned about the Australasian and Afrotropical fauna (Burla 1954, Mather 1954; Tsacas 1979; 1980). Currently, the seven species groups placed in *Sophophora* contain approximately 300 species (Wheeler 1982; 1986; Lemeunier, *et al.* 1986).

Throckmorton (1975) considered species placed in the subgenus *Sophophora* to be part of a large radiation of flies which also contained the genera *Chymomyza* and *Neotanygastrella*. He considered the "Sophophoran radiation" to be basal to the radiation which gave rise to the remainder of the genus *Drosophila* (Throckmorton 1975). Recent molecular studies suggest, however, that the subgenus *Sophophora* is actually a sister clade to the genus *Drosophila* and not closely related to *Chymomyza* or *Neotanygastrella* (DeSalle 1992; Russo, *et al.* 1995).

Based on biogeographical data, a common ancestral "*protomelanogaster*" lineage gave rise to both the *melanogaster* and *obscura* species groups in southeast Asia during the mid-Oligocene (Throckmorton 1975). The *melanogaster* species group consists of predominantly Old World tropical species, although some members are cosmopolitan in distribution (Lemeunier, *et al* 1986; Lachaise, *et al.* 1988). Although the *obscura* species group is found primarily in the Holarctic region, some members are found in tropical regions (Lakovaara and Saura, 1982). The *saltans* and *willistoni* species groups comprise a clade of Neotropical species, closely related to one another, but distinct from all other Sophophoran forms (Throckmorton 1975). Throckmorton (1975) considered the *saltans* and *willistoni* species groups to be derivative within the subgenus, originating after the divergence of the *melanogaster* and *obscura* species groups from the "*protomelanogaster*" ancestor.

Currently, there are two competing hypotheses for relationships within *Sophophora*. Morphological studies and phylogenetic analysis of nucleotide sequences from the mt *16S* and nu *Adh* loci have indicated that the subgenus *Sophophora* is monophyletic, as are its four largest species groups, *melanogaster*, *obscura*, *saltans*, and *willistoni* (Throckmorton 1975; DeSalle 1992; Thomas and Hunt 1993; Russo, *et al.* 1995). Furthermore, these data also support the monophyly of the Old World *melanogaster-obscura* and Neotropical *saltans-willistoni* clades (Fig. 1A). In these

studies, the subgenus *Sophophora* was always monophyletic with respect to both the subgenus *Drosophila* and several genera within the family Drosophilidae.

Pelendakis, *et al.* (1991) and Pelendakis and Solignac (1993) present a very different hypothesis based on neighbor joining analysis of the *28S* rRNA locus. Their results indicate that the *melanogaster* species group, as traditionally defined, is not a monophyletic lineage. The *ananassae* subgroup, which has traditionally been placed in the *melanogaster* species group, was shown to be the sister taxon of the *obscura* species group (Fig. 1B). However, it should be noted that bootstrap support for this relationship is not high. The *fima* species group is the sister group to the *obscura-ananassae* clade (Fig. 1B). The *saltans* species group was monophyletic in this study, as was the *saltans-willistoni* clade, but the *willistoni* species group is paraphyletic with respect to the *saltans* species group (Fig. 1B). The result that was most incongruent with the previous morphological and molecular studies, however, was the finding that *Sophophora* was paraphyletic with respect to the subgenus *Drosophila*. The *28S* data support the notion that the *obscura-melanogaster* clade is the sister taxon of the subgenus *Drosophila*, not the Neotropical *saltans* and *willistoni* species (Fig. 1B). This result is not only incongruent with other nucleotide sequence data (DeSalle 1992; Thomas and Hunt 1993; Russo, *et al.* 1995), but also with previous morphological and biogeographical studies (Throckmorton, 1975; Lemeunier, *et al.* 1986; Lachaise, *et al.* 1988).

We have gathered additional molecular sequence data from the mt *COII* and nu *Adh* genes and examine it, along with previously published data from the *28S* rRNA locus (Pelendakis, *et al.* 1991; Pelendakis and Solignac 1993), in both individual and combined analyses to test these two competing theories (Figs. 1A and 1B). Primarily, we are interested in determining (1) if the major clades defined within the subgenus *Sophophora* (the *melanogaster*, *obscura*, *saltans* and *willistoni* species groups, the *saltans-willistoni* clade and the *melanogaster-obscura* clade) are monophyletic and (2) if the subgenus *Sophophora* itself is monophyletic.

Materials and Methods

DNA Sources, Isolation, and Sequencing

Live *Drosophila* stocks were obtained from the National *Drosophila* Species Resource Center in Bowling Green, Ohio. Cultures of *D. madeirensis* were obtained from D. Sperlich at the University of Tubingen. Specimens of *D. obscura* were obtained from M. Radak at the University of Belgrade. Table 1 shows the taxonomic classification of the species used in this study along with the Genbank accession numbers for each sequence.

Genomic DNA was isolated following the method of Gloor and Engels (1992). The target loci were amplified from each taxon using standard PCR cycling conditions. Oligonucleotides 4682 ACAT(TC)CAGCCAIGAGTTGAA(CT)TTGTG, located in the

first exon, and 4683 CTGGGIGGCATTGGI(CT)T(CG)GACACCAC, located in the third exon, were used to amplify the *Adh* gene. PCR products from the *Adh* gene were then cloned into the TA cloning vector (Invitrogen). Two single colonies were selected and sequenced using a dsDNA cycle sequencing procedure (GIBCO-BRL). When possible, both DNA strands were sequenced from multiple clones to reduce the effect on polymerase error on the analysis. Oligonucleotides, designed after Simon, *et al.* (1994), used to amplify the entire 688 bp *COII* gene were (1) ATGGCAGATTAGTGCAATGG and (2) GTTTAAGAGACCAGTACTTG. These products were purified by membrane filtration (Millipore) and sequenced directly using a standard dsDNA cycle sequencing protocol (GIBCO-BRL). Approximately 90% of both DNA strands were sequenced from each PCR product.

Sequence Alignment

Alignment of the *28S* locus was exactly as in Pelendakis, *et al.* (1991) and Pelendakis and Solignac (1993). The alignment of the *COII* and *Adh* coding regions was trivial. One gap, following nucleotide number 685 at the 3' end of the *COII* gene was required to align the *melanogaster* species group to the other taxa in this study.

Small Individual Analyses

Thirteen taxa were common to the *28S*, *Adh*, and *COII* data sets. These species were analyzed in individual and combined analyses with PAUP* 4.0d63 (Swofford 1998). Individual analyses were performed using a variety of optimality criteria, including maximum likelihood (ML), neighbor joining (NJ) and maximum parsimony (MP). We present the results of ML and MP searches for the individual analyses (Fig. 2). Individual MP searches were performed with the branch and bound algorithm. The level of support at each node of all most parsimonious trees obtained was assessed using decay indices (DI; Bremer 1988) and bootstrap proportions (BP; Felsenstein 1985; 1988). Five hundred replicates were performed in each bootstrap search. Individual ML analyses employed the following simple model of evolution: (1) nucleotide frequencies were determined empirically from the data, (2) all sites were assumed to evolve at the same rate, and (3) the transition/transversion ratio was set to 2 (Hasegawa, *et al.* 1985). Support for each clade in the ML trees was determined using bootstrap proportions (Felsenstein 1985; 1988). One hundred replicates were performed for each locus. Since the ML and MP tree topologies were identical, only the MP trees are presented along with likelihood scores from ML analyses. All trees were rooted using the genera *Chymomyza* and *Scaptodrosophila*.

Large Individual Analyses

All sequences determined for each individual locus were analyzed using maximum parsimony. Heuristic searches were performed (starting trees obtained via random stepwise addition; 500 replicates; TBR branch swapping employed) to find the most parsimonious trees. Strict consensus trees, with bootstrap proportions (of 200 replicates) and decay indices are shown in Figs. 3A - 3C. Outgroups were *Chymomyza* and *Scaptodrosophila.*

Partition Homogeneity Test

The partition homogeneity test (PHT; Farris, *et al.* 1994; 1995), as implemented in PAUP* 4.0d63 (Swofford 1998) was used to test for incongruence between data sets. The null hypothesis of the PHT is that each pair of loci are as congruent as two randomly generated partitions of equal size. The test compares the length of the most parsimonious tree(s) for the original pair of partitions with a number of randomly generated data sets. The character columns in the random data sets are rearranged, but size of each partition is kept constant. One hundred randomly generated data sets were used to create a null distribution to test the statistical significance of treelengths from the original partitions. We performed pairwise PHTs to test for incongruence on two sets of taxa. Table 2 shows the results of the PHT on those species used in the small individual and combined analyses. Table 3 shows the PHT results from those taxa used in the pairwise combined analyses.

Combined Analyses

Only MP was used to analyze combined data partitions. Three classes of combined analyses were performed, (1) the small combined analysis (Fig. 2) which contains only those taxa which have sequences determined for *28S*, *Adh*, and *COII*, (2) the pairwise combined analyses (Figs. 5A - 5C) which consist of the largest sets of taxa which have sequences determined for any two loci, and (3) the large combined analysis (Fig. 6) which include those taxa which have two or more of the three loci in this study determined. Combined analyses on the small data set and the pairwise analyses were performed using only the branch and bound, maximum parsimony algorithm implemented in PAUP* 4.0d63 (Swofford 1998). The large pairwise analysis was performed using heuristic searches (starting trees obtained via random stepwise addition; 500 replicates; TBR branch swapping employed) to find the most parsimonious trees. Level of support for each node was calculated as in the individual analyses. All trees were rooted using the genera *Chymomyza* and *Scaptodrosophila*.

We also used partitioned Bremer support (Baker and DeSalle 1997) to measure the amount of support provided by each individual partition to the DI for every node in the combined analysis phylogenies (Figs. 4-6). Partitioned Bremer support (PBS) shows the contribution of each partition to the decay index of every node on the total evidence tree. To obtain the PBS value for a given node on the total evidence tree, the length of the

partition on the unconstrained total evidence tree was subtracted from the length of a partition on a tree constrained to not contain the node of interest. If the partition supports a relationship represented by a node in the total evidence tree, then the PBS value will be positive. If, on the other hand, a partition supports an alternative relationship, the PBS value will be negative, indicating incongruence with the simultaneous analysis. The magnitude of PBS values indicate the level of support for, or incongruence with, a node. The sum of all partition lengths for any given node will always equal the decay index for that node on the total evidence tree. Using this method allows us to determine the relative contribution of each partition to the different simultaneous analysis trees (Figs. 4-6).

Results

Small Individual Analyses

The thirteen species for which all three loci were sequenced were analyzed in both individual and combined analyses to estimate the phylogeny of the subgenus *Sophophora* (Figs. 2 and 4). The individual nu *28S* analysis (Fig. 2A) indicates that the *melanogaster*, *obscura*, *saltans* and *willistoni* species groups were monophyletic. However, with the exception of the New World *saltans* and *willistoni* species groups being sister taxa, relationships among the four species groups in this analysis are not resolved (Fig. 2A).

Figure 2B shows the results of the individual analysis based on the coding regions of the nu *Adh* gene. While the *Adh* locus shows that the *saltans* and *willistoni* species groups form a clade, it is unable to resolve a monophyletic *willistoni* species group (Fig. 2B). The *melanogaster*, *obscura*, and *saltans* species groups are monophyletic in this analysis, but relationships among the Old World species groups and the New World clade are unresolved.

The results of the individual analysis of the mt *COII* gene are shown in Fig. 2C. Relationships among the four species groups are mostly unresolved. The *saltans* and *willistoni* species groups do form a clade, but relationships within the *saltans* species group are completely unresolved and there is weak support (BP = 56/54; DI = 1) for a paraphyletic *willistoni* species group. *D. eugracilis*, a species typically placed in the *melanogaster* species group, is unresolved with respect to the *obscura* species group, the *saltans-willistoni* clade, and the remained of the *melanogaster* species group.

Large Individual Analyses

The individual analysis of the nu *28S* locus is shown in Fig. 3A. Parsimony analysis indicates that relationships among the five *Sophophora* species groups, as well as among the seven *melanogaster* species subgroups, are largely unresolved. Within the *melanogaster* species group, the *melanogaster* and *montium* subgroups are monophyletic, although support for their monophyly is not strong (BP = 64, DI = 2 and BP = 55, DI =

1, respectively). The *melanogaster* subgroup species are mostly unresolved, but there is support for the closely related triad of *D. simulans*, *D. mauritiana*, and *D. sechellia* (Lachaise, *et al.* 1988). Phylogenetic relationships within the *montium* subgroup are completely unresolved. The *ananassae* subgroup is not monophyletic with respect to the *fima* species group, but there is weak support (BP = < 50, DI = 1) for these groups forming a clade. There is some support for the sister group relationships between the *suzukii* and *takahashii* (BP = 59, DI = 1) and *ficusphila* and *elegans* (BP = 80, DI = 2) species groups. There is strong support for the *melanogaster*, *eugracilis*, *suzukii* and *takahashii* subgroups forming a clade (BP = 90, DI = 4) but, with the exception of the *suzukii-takahashii* relationship, there is no resolution among these groups. The three *obscura* species sampled form a clade (BP = 98, DI = 4), but relationships within this group, as well as relationships between the *obscura* species group and the other Sophophoran species groups, are unresolved. The New World *saltans* and *willistoni* species groups form a poorly supported (BP < 50, DI = 1) monophyletic group (Fig. 3A). Within this clade, the *saltans* group is weakly supported as monophyletic (BP = 59, DI = 1) and the *willistoni* species are unresolved (Fig. 3A).

Figure 3B is the result of parsimony analysis on the *Adh* gene. The *melanogaster* and *obscura* groups form a monophyletic group (BP = 92, DI = 4). The *melanogaster* species group is also monophyletic, (BP = 99, DI = 5), with the *montium*, *eugracilis* and *melanogaster* subgroups forming an unresolved trichotomy at the base of this clade (Fig.

3B). The *obscura* species group is also monophyletic (BP = 95, DI = 5). Within the *obscura* group, the *subobscura* and *pseudoobscura* subgroups are also monophyletic (BP = 100, DI = 8 and BP = 100, DI = 25, respectively). The relationships among *D. guanche*, the single representative of the *obscura* subgroup, and the *subobscura* and *pseudoobscura* subgroups are unresolved. The *saltans* and *willistoni* species groups form a well supported (BP = 98, DI = 12) monophyletic group (Fig. 3B). Within the *saltans-willistoni* clade, there is weak support for a monophyletic saltans group (BP = 74, DI = 3) and no support for a monophyletic *willistoni* group. The relationships within each of the Neotropical species groups are not well resolved (Fig. 3B).

Results of the maximum parsimony analysis of the *COII* gene are shown in Fig. 3C. This locus also supports, albeit weakly (BP <50, DI = 1), the *melanogaster* and *obscura* sister group relationship. The *montium* subgroup is weakly supported as the sister clade of the rest of the *melanogaster* species groups (Fig. 3C). The remaining subgroups in the *melanogaster* species group are not well resolved. Within the monophyletic *obscura* species group, the *affinis* and *pseudoobscura* subgroups are monophyletic sister taxa (Fig. 3C). The *subobscura* subgroup is also monophyletic and nested within a paraphyletic *obscura* subgroup (Fig. 3C). There is also weak support (BP <50, DI = 3) for a *saltans-willistoni* clade in this analysis. The *willistoni* species group is monophyletic (BP = 68, DI = 4) and relationships within this group of closely related species are better resolved than in the previous two analyses (Figs. 3A and 3B).

The *saltans* species group is not monophyletic, with the *parasaltans* subgroup being weakly supported (BP <50, DI = 2) as the sister group of the remainder of the *saltans* species and the *willistoni* species group (Fig. 3C). Relationships within the *saltans* group are not well supported by bootstrap proportions.

Partition Homogeneity Tests

The results of the PHT on the small individual data sets is shown in Table 2. Each locus was compared to the other two loci in this study and none were significantly incongruent. The PBS values correspond well with this finding. Most PBS values are either positive or zero, indicating support for a node or lack of conflict (Figs4–6). The results of the PHT on the pairwise combined data sets is shown in Table 3. The *COII* and *Adh* loci were shown to be significantly incongruent. Examining the PBS values for the *Adh-COII* pairwise combined analysis (Fig.5C), we can see that the areas of most incongruence between these two loci is within the *saltans* and *willistoni* species groups. This conflict leads to poorly supported relationships in these clades. Those combined partitions which show no sign of incongruence have mostly non-negative PBS values, indicating that there is support (or lack of conflict) from each locus at every node (Fig. 5C).

Small Combined Analysis

The results of the small combined analysis of the *28S*, *Adh* and *COII* loci are shown in Fig. 4. The Old World ***melanogaster*** and ***obscura*** species groups are monophyletic sister taxa, a result not seen in any of the individual analyses. This phylogeny indicates that the ***melanogaster*** complex (Lemeunier, et al. 1986), represented here by *D. **melanogaster**, D. **simulans*** and *D. **mauritiana***, is monophyletic and the sister clade of the ***yakuba*** complex, represented by *D. **yakuba***. The ***saltans*** and ***willistoni*** species groups also form a clade (Fig. 4). The ***saltans*** species group is monophyletic, but relationships within this group are not well resolved. This combined analysis is unable to resolve a monophyletic ***willistoni*** species group.

Combined Analyses - Pairwise Searches

Pairwise analyses were performed on the largest sets of taxa which had two sequences determined (i.e., *28S* and *Adh*, *28S* and *COII*, *Adh* and *COII*). Figure 5A shows the results of combined analysis of the *28S* and *Adh* loci for the taxa that are common to both data sets. The ***melanogaster*** and ***obscura*** species groups are monophyletic sister taxa (BP = 57, DI = 2). The ***saltans*** and ***willistoni*** species groups form a well supported clade (BP = 100, DI = 20), but the ***willistoni*** species group is paraphyletic and phylogenetic relationships within the ***saltans*** species group are poorly supported.

The results of parsimony analysis on the taxa in common to the *28S* and *COII* data sets are shown in Fig. 5B. The ***melanogaster*** species group comes out in two

monophyletic groups, the *ananassae* subgroup and the *melanogaster-ficusphila-suzukii-eugracilis* clade (Fig. 5B). This analysis is unable to determine if they are sister taxa or instead, if one is more closely related to the *pseudoobscura* species group or *saltans-willistoni* clade (Fig. 5B). The three *obscura* group species examined here are monophyletic and well resolved. The *saltans* and *willistoni* species groups are monophyletic sister taxa (BP = 83, DI = 3).

The results of phylogenetic analysis of the taxa common to both the *Adh* and *COII* genes is shown in Fig. 5C. This analysis indicates strong support for the *melanogaster-obscura* (BP = 100, DI = 13) and the *saltans-willistoni* (BP = 100, DI = 31) clades. Within the *melanogaster-obscura* clade, both the *melanogaster* and *obscura* species groups are monophyletic and relationships among species in each group are well resolved and congruent with previously published data (Fig. 5C). The *saltans* species group is also monophyletic, but relationships among the five subgroups examined in this study are mostly unresolved. Two *willistoni* species, *D. fumipennis* and *D. nebulosa*, are unresolved with respect to the *saltans* group, indicating that the *willistoni* group may not be monophyletic (Fig 5C). The remaining species in the *willistoni* group are resolved, but support for some nodes is weak.

Large Combined Analysis

Figure 6 shows the results of phylogenetic analysis of those taxa which have sequences for at least two of the three loci examined in this study. The subgenus *Sophophora* is monophyletic (BP = 79 , DI = 1) with respect to the four subgenus *Drosophila* species groups included in this analysis (Fig. 6). The *melanogaster* and *obscura* species groups form a clade (Fig. 6). This combined analysis was unable to determine if the *melanogaster* species group is monophyletic. The *ananassae* subgroup is unresolved with respect to the remaining *melanogaster* subgroups and the *obscura* species group. The *montium* subgroup seems to be basal to the remaining *melanogaster* subgroups (Fig. 6). This analysis also suggests that the *ficusphila*, *eugracilis*, *suzukii*, and *takahashii* subgroups are basal to the African *melanogaster* subgroup. The *obscura* species group is monophyletic (BP = 86, DI = 5), as are the four species subgroups examined within the *obscura* species group. The *affinis* and *pseudoobscura* subgroups form a clade, as do the *obscura* and *subobscura* subgroups. This analysis indicates that the *saltans* and *willistoni* species groups form a well supported (BP = 93, DI = 7) Neotropical clade. The *willistoni* subgroup is weakly supported as monophyletic (BP <50, DI = 4), but phylogenetic relationships within this recently derived species group are mostly unresolved, even though the "subgroup B" species form a monophyletic group (Fig. 6). The *saltans* species group is also monophyletic (BP = 77, DI = 6) and relationships within the *saltans* subgroup are resolved, but relationships among the *saltans*, *parasaltans*, *elliptica*, *cordata* and *sturtevanti* subgroups are not well supported.

Discussion

The relationships of species within the subgenus *Sophophora* have been studied using both morphological (Throckmorton 1975) and molecular characters (Pelendakis, *et al.* 1991; DeSalle 1992; Pelendakis and Solignac 1993, Thomas and Hunt 1993; Russo, *et al.* 1995). The results of some of these studies are incongruent with one another, primarily concerning the monophyly of the subgenus *Sophophora* and the monophyly of the major species groups placed within *Sophophora*. Morphological data, along with mt *16S* and nu *Adh* DNA sequences, support the monophyly of the subgenus *Sophophora* and the monophyly of the *melanogaster*, *obscura*, and *willistoni* species groups within this subgenus (Throckmorton 1975; DeSalle 1992; Thomas and Hunt 1993; Russo, *et al.* 1995). Analysis of sequences from the nu *28S* locus, however, suggest that the subgenus *Sophophora* and the *melanogaster* and *willistoni* species groups are not monophyletic (Pelendakis, *et al.* 1991; Pelendakis and Solignac 1993).

Incongruence between these phylogenetic hypotheses could have several explanations, including different sampling strategies (Lecointre, *et al.* 1993), different methods of phylogeny reconstruction (Huelsenbeck and Hillis 1993), or different histories for each locus (Pamilo and Nei 1988; Hudson 1990; Bull, et al. 1993). Our analyses, which increase the number of taxa sampled for the mt *COII* and nu *Adh* loci and employ a maximum parsimony algorithm to analyze all the data suggests little

incongruence among partitions. Only the *Adh vs. COII* comparison in the pairwise analysis is significantly incongruent by a PHT. The cause of this incongruence is due to conflicting relationships within the *saltans* and *willistoni* species groups and is manifested in low BPs and DIs. Interestingly, *COII* and *Adh* do not conflict in the small PHT comparison, so incongruence in this case seems to be sensitive to the number of taxa being compared.

The subgenus *Sophophora*

The large combined analysis included representatives from several species groups (*immigrans*, *quinaria*, *repleta*, and *virilis*) placed in the subgenus *Drosophila* to specifically test the monophyly of the subgenus *Sophophora*. Combined analysis of the *28S*, *Adh* and *COII* sequences suggest that *Sophophora* is monophyletic (BP = 70, DI = 1) relative to the genus *Drosophila* (Fig. 6), which is also monophyletic (BP = 91, DI = 8). This result agrees with Throckmorton's (1975) taxonomic and biogeographic studies and previously published molecular data (DeSalle 1992; Thomas and Hunt 1993; Russo, et al. 1995).

The *Obscura-melanogaster* Clade

The *obscura-melanogaster* clade (Pelendakis, *et al.* 1991; Pelendakis and Solignac 1993) is monophyletic in several of the analyses in this study, including the large

individual *Adh* (Fig. 3B; BP = 92, DI = 4) and *COII* (Fig. 3C; BP < 50, DI = 1) analyses, the small combined analysis (Fig. 4; BP = 62, DI = 3), the *28S&Adh* (Fig. 5A; BP = 57, DI = 2) and *Adh&COII* (Fig. 5C; BP = 100, DI = 13) pairwise analyses, and the large combined analysis (Fig. 6; BP = 61, DI = 1). This clade is not resolved as being monophyletic in any of the small individual analyses (Fig 2A - 2C), the large *28S* analysis (Fig. 3A), or the *28S&COII* pairwise analysis (Fig. 5B). Examining PBS values from combined analyses, we see that the *Adh* and *COII* genes are giving the most support to the monophyly of the Old World *obscura-melanogaster* clade (Figs. 4-6). PBS values for the *28S* locus are zero or greater in all combined analyses, indicating that while these characters do not strongly support this clade, they do not conflict with it either. The only partition which contributes a negative PBS value to the *obscura-melanogaster* clade in combined analyses is *COII* (PBS = - 1), but only in the small combined analysis (Fig. 4). This may be due to the poor species sampling in the small combined analysis relative to the larger combined analyses (Figs. 5C and 6). Overall, our results agree with the previous studies (Pelendakis, *et al.* 1991; DeSalle 1992; Pelendakis and Solignac 1993; Russo, *et al.* 1995) and find that the *obscura-melanogaster* clade is monophyletic (Fig. 6).

The *Drosophila melanogaster* species group

The *melanogaster* species group was monophyletic in several of the analyses in this study. However, in the case of the small individual (Figs. 2A - 2C) and combined

analyses (Fig. 4), many of the *melanogaster* species group sequences examined were from very closely related species, indicating that perhaps the monophyly of the larger group was not tested rigorously enough. We should focus, instead, on those analyses which sample taxa from more disparate groups within the *melanogaster* species group, such as the large individual analyses (Fig. 3), the *28S&COII* and *Adh&COII* pairwise analyses (Fig. 5B and 5C), and the large combined analysis (Fig. 6). The analyses which sample more broadly from the *melanogaster* species group indicate that this clade is not resolved as monophyletic.

Morphological and cytological data agree with some of the findings in the present study. For example, Bock and Wheeler (1972) proposed that the *ananassae* and *montium* species groups diverged from the other subgroups early in the evolution of the *melanogaster* species group. This is what we see in Fig. 6. The *ananassae* subgroup is unresolved with respect to the *obscura* species group and the remainder of the *melanogaster* subgroups (Fig. 6), indicating that perhaps it diverged soon after the *protomelanogaster* split. The *montium* subgroup is also located at the base of the *melanogaster* species group (Fig. 6). Ashburner, *et al.* (1984) used polytene chromosome banding patterns to suggest the following clades: (1) *ananassae*, (2) *montium*, and (3) *suzukii- takahashii-ficusphila-melanogaster-eugracilis*. These series correspond well with relationships in the large combined analysis (Fig. 6).

The *Drosophila obscura* species group

The *obscura* species group is strongly supported as monophyletic in all analyses in this study which sampled more than a single *obscura* group taxon. PBS support for the *obscura* group in combined analyses comes equally from each locus in most combined analyses (*e.g.*, Figs. 5B and 5C). No locus in any analysis contributes negatively to the combined decay index for the *obscura* species group. Within the *obscura* group the large *COII* analysis (Fig. 3C) and the large combined analysis (Fig. 6) agree that the New World *pseudoobscura* and *affinis* subgroups are sister groups. Furthermore, the large *COII* analysis, which samples more *obscura* subgroup species than the other analyses in this study, suggests that the *subobscura* subgroup is monophyletic and nested within a paraphyletic *obscura* subgroup (Fig. 3C). This is congruent with other phylogenetic analyses of the *obscura* species group (Beckenbach, *et al.* 1993; Barrio, *et al.* 1994; Barrio and Ayala 1997; Gleason, *et al.* 1997; O'Grady and Heed, unpublished).

The *Saltans-willistoni* Clade

The Neotropical *saltans-willistoni* clade is monophyletic in every analysis in the present study, usually with high bootstrap proportions and decay indices. The PBS values calculated for the combined analyses performed indicate that, with the exception of *Adh* (PBS = 0) in the large combined analysis (Fig. 6), all loci contribute positively to the decay index at the node supporting the monophyly of the *saltans-willistoni* lineage. The

Neotropical clade is, according to these analyses and previous studies, a well supported monophyletic group (Throckmorton 1975; Pelendakis, *et al.* 1991; Pelendakis and Solignac 1993).

The *Drosophila saltans* species group

Only the large individual *COII* analysis indicates that the *saltans* group may not be monophyletic (Fig. 3C). This analysis places *D. subsaltans*, a member of the *parasaltans* subgroup, as the sister taxon of all species in the *saltans* and *willistoni* groups. However, this may not be a robust result, as the bootstrap proportions which support this grouping are all less than 50 percent. All other analyses in the present study support a monophyletic *saltans* species group. Support, in the sense of PBS values, comes from all loci in most of the combined analyses (Figs. 4-6). Relationships among species in the *saltans* group are variable, depending on the gene examined and the taxa included. The large combined analysis (Fig. 6), as well as other combined analyses which sample more than a single species from each subgroup in the *saltans* group (*e.g.*, Fig. 5C), are able recover monophyletic *saltans* and *sturtevanti* subgroups, but cannot resolve relationships among any of the subgroups. More extensive species and sequence sampling is better able to resolve relationships within this species group (O'Grady, *et al.* 1998). The data in the present study indicate that the *saltans* species group is

monophyletic, although relationships among subgroups within this species group should be considered tentative.

The *Drosophila willistoni* species group

Several analyses in this study are unable to recover a monophyletic *willistoni* species group (Figs. 2B, 2C, 3A, 3B, 4, 5A, and 5C). Those analyses which do suggest that this group is monophyletic are not well supported (BP < 70; Figs. 2A, 3C, 5B, and 6). Within the *willistoni* group, relationships are not well resolved in any analyses. The two proposed subgroups, A and B (Wheeler 1949; Wheeler and Magalhaes 1962), in the *willistoni* species group are also not well supported as monophyletic. However, the large combined analysis indicates, with low support, that the *willistoni* species group is monophyletic (BP < 50, DI = 4) and the subgroups A and B are monophyletic sister taxa (BP = 63, DI = 2 and BP < 50, DI = 2, respectively). This should be taken as a provisional hypothesis of relationships within the *willistoni* species group pending further analyses.

Conclusions

The conflicting results presented in previous studies (Pelendakis, *et al.* 1991; DeSalle 1992; Pelendakis and Solignac 993; Thomas and Hunt 1993; Russo, *et al.* 1995) are somewhat reconciled by the analyses presented here. The reanalysis of the *28S* data,

along with additional sequences from the *Adh* and *COII* genes indicates that *Sophophora* is monophyletic. Furthermore, the *obscura-melanogaster* and *saltans-willistoni* clades within this subgenus are also monophyletic. The results presented here also indicate that the *obscura* and *saltans* species groups are monophyletic. However, it seems that the *melanogaster* and *willistoni* species groups are either not, or only weakly supported, as monophyletic. Additional morphological and molecular data from other species closely related to both the ananassae subgroup and D. fumipennis and D. nebulosa will be required to fully resolve these realtionships. It is possible that the *ananassae* subgroup diverged very early within the *obscura-melanogaster* clade, making it difficult to determine whether it forms a clade with the remaining species in the *melanogaster* species group or is, instead, the sister clade to the *obscura* species group. Sampling additional representatives of the *ananassae* subgroup, as well as species from other closely related clades, such as the *montium* subgroup, may clarify the affinities of this clade. The situation within the *willistoni* species group is similar; *D. fumipennis* and *D. nebulosa* are closely related to the *bocainensis* subgroup (Wheeler 1949), a clade of poorly known species which are currently unavailable for sampling. Representatives of the bocainensis subgroup should be collected and included in an expanded phylogenetic analysis to fully understand how this group evolved.

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Table 1

Taxonomic Classification of *Sophophora* and Genbank Accession Numbers of Taxa in the Present Study

				Genbank Accession Number		
Species Group	Subgroup	Species	BG No.	*28S*	*Adh*	*COII*
fima		*D. fima*	N/A	??	--	--
melanogaster	*ananassae*	*D. ananassae*	14024-0371.0	--	--	XX
		D. malerkotliana	14024-0391.1	--	--	XX
		D. vallismaia	N/A	??	--	--
		D. varians	N/A	??	--	--
	elegans	*D. elegans*	N/A	??	--	--
	eugracilis	*D. eugracilis* (EUG)	14026-0451.0	??	XX	XX
	fiscuphila	*D. ficusphila*	14025.0441.0	??	--	XX

Table 2

Results of Partition Homogeneity Test (Small Combined Data Sets)

Locus	*28S*	*Adh*	*COII*
28S	-	0.42	0.57
Adh		-	0.89
COII			-

Table 3

Results of Partition Homogeneity Test (Pairwise Combined Data Sets)

Locus	*28S*	*Adh*	*COII*
28S	-	0.33	0.87
Adh		-	0.01*
COII			-

* Data partitions which display significant homogeneity when compared

Figure Legends

FIG. 1. - Proposed evolutionary relationships within the subgenus *Sophophora*. (A) Evolutionary scenario supported by morphology, biogeography and molecular mt *16S* and nu *Adh* data. (B) Evolutionary scenario supported by analysis of nu *28S* rRNA.

FIG. 2. - Results of small individual analyses. Each phylogeny is shown with the length, in base pairs (bp) of the partition, the number of parsimony informative characters (PICs) in the data set, the number of most parsimonious trees (MPTs) found, the number of steps on each MPT, the ensemble retention (RI) and consistency (CI) indices, and the likelihood score (-Ln). MP bootstrap proportions are above the node to the left, ML bootstrap proportions are above the node to the right, and decay indices are shown below each node. (A) *28S*. (B) *Adh*. (C) *COII*.

FIG. 3. - Results of large individual analyses. Each phylogeny is shown with the length, in base pairs (bp) of the partition, the number of parsimony informative characters (PICs) in the data set, the number of most parsimonious trees (MPTs) found, the number of steps on each MPT, and the ensemble retention (RI) and consistency (CI) indices. Bootstrap proportions are above the node and decay indices are shown below each node. (A) *28S*. (B) *Adh*. (C) *COII*.

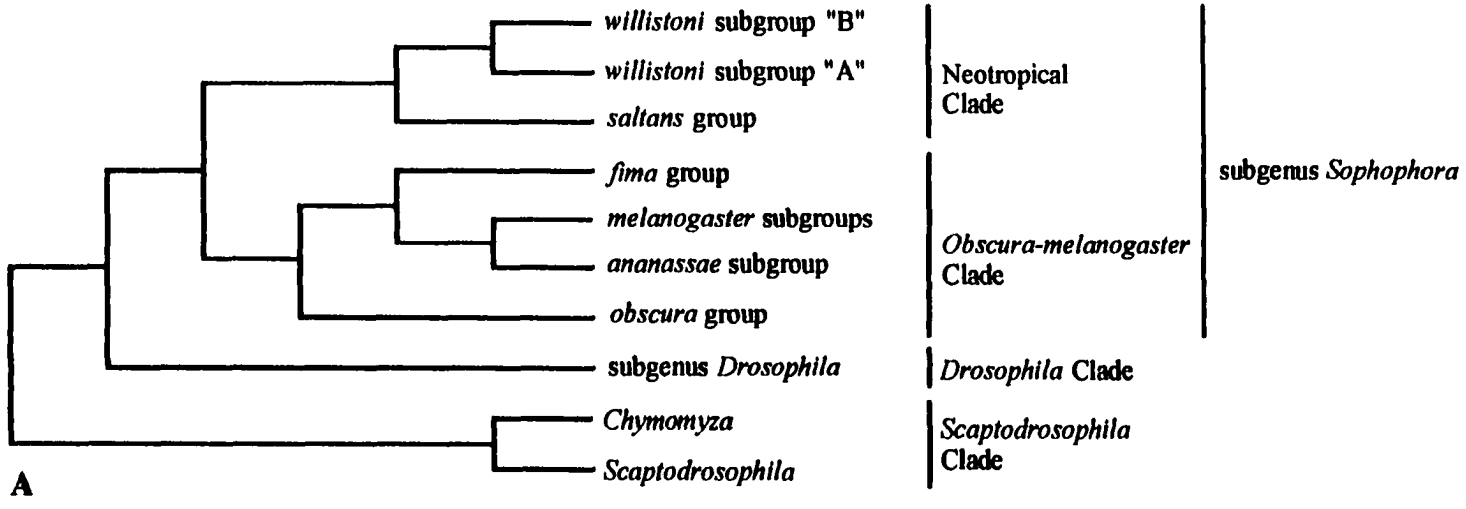
FIG. 4. - Results of the small combined analysis. Each phylogeny is shown with the length, in base pairs (bp) of the data set, the number of parsimony informative (PI) characters in the data set, the number of most parsimonious trees (MPTs) found, the number of steps on each MPT, and the ensemble retention (RI) and consistency (CI) indices. BPs (above node), Dis (below node), and PBS values (in boxes at node) are indicated. Branch lengths are not proportional to change, they vary only for clarity.

FIG. 5. - Results of combined pairwise analyses. Each phylogeny is shown with the length, in base pairs (bp) of the data set, the number of parsimony informative (PI) characters in the data set, the number of most parsimonious trees (MPTs) found, the number of steps on each MPT, and the ensemble retention (RI) and consistency (CI) indices. BPs (above node), Dis (below node), and PBS values (in boxes at node) are indicated. Branch lengths are not proportional to change, they vary only for clarity. (A) *28S&Adh.* (B) *28S&COII.* (C) *Adh&COII.*

FIG. 6. - Results of large combined analysis. The phylogeny is shown with the length, in base pairs (bp) of the partition, the number of parsimony informative (PI) characters in the data set, the number of most parsimonious trees (MPTs) found, the number of steps on each MPT, and the ensemble retention (RI) and consistency (CI) indices. BPs (above

node), Dis (below node), and PBS values (in boxes at node) are indicated. Branch lengths are not proportional to change, they vary only for clarity.

Figure 1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saltans group
fima group
melanogaster subgroups
ananassae subgroup
obscura group
subgenus *Drosophila*
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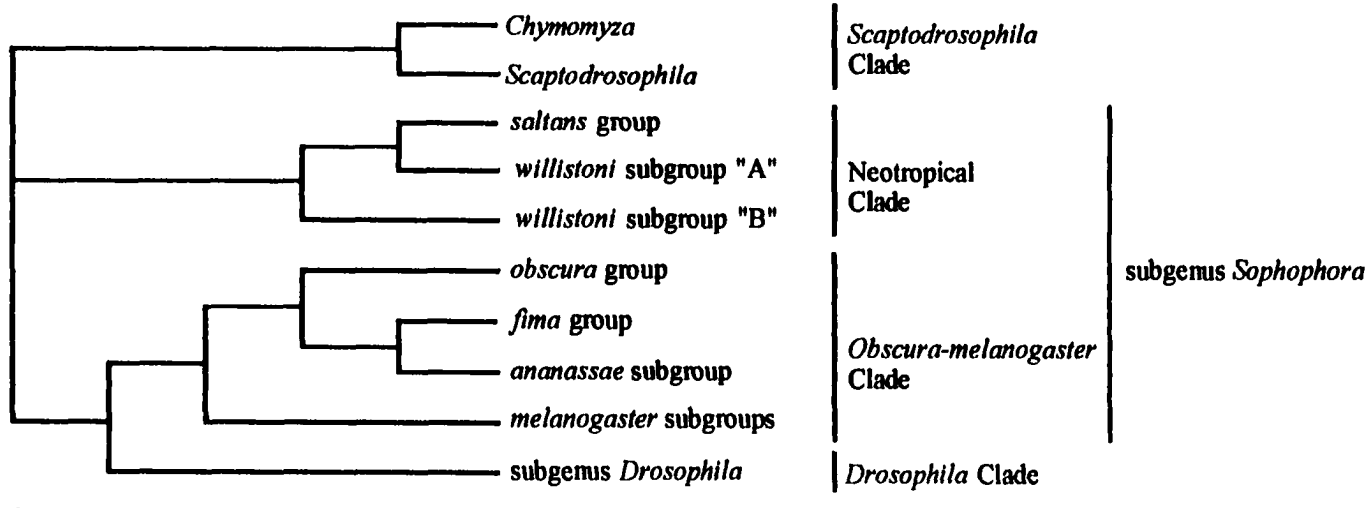
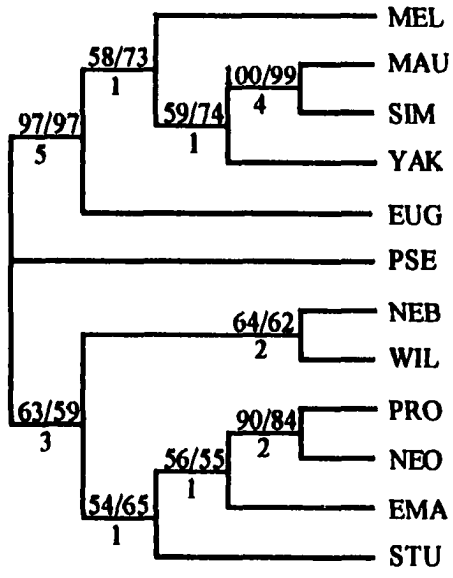
Scaptodrosophila
Neotropical Clade
Obscura-melanogaster Clade
Drosophila Clade
Scaptodrosophila Clade
subgenus *Sophophora*

A

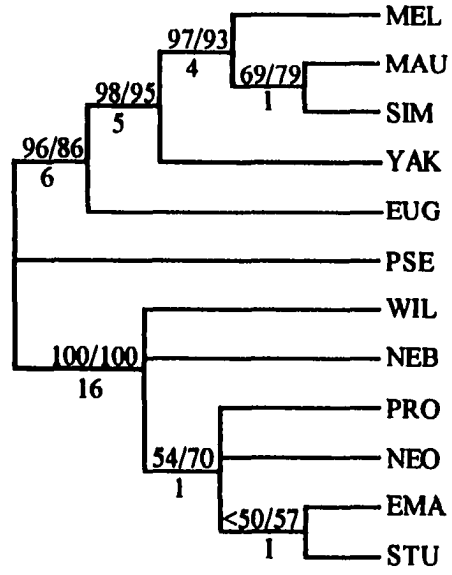

Chymomyza
Scaptodrosophila
saltans group
willistoni subgroup "A"
willistoni subgroup "B"
obscura group
fima group
ananassae subgroup
melanogaster subgroups
subgenus *Drosophila*
Scaptodrosophila Clade
Neotropical Clade
Obscura-melanogaster Clade
Drosophila Clade
subgenus *Sophophora*

B

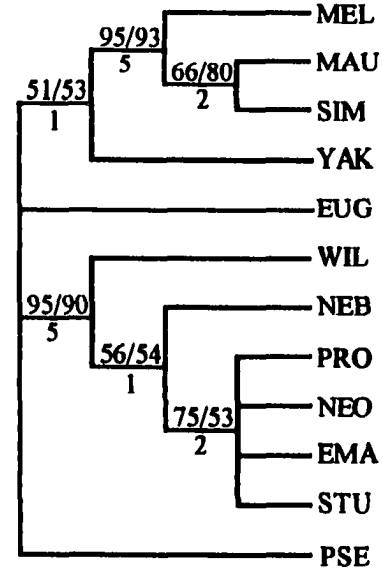
Figure 2



A. *28S*
Maximum Parsimony
1 MPT, 101 Steps, 34 PICs
CI = 0.822, RI = 0.800
Maximum Likelihood
-ln likelihood = 1008.33764



B. *Adh*
Maximum Parsimony
3 MPTs, 492 Steps, 169 PICs
CI = 0.754, RI = 0.730
Maximum Likelihood
-ln likelihood = 3379.54494



C. *COII*
Maximum Parsimony
6 MPTs, 425 Steps, 119 PICs
CI = 0.593, RI = 0.488
Maximum Likelihood
-ln likelihood = 3152.40237

Figure 3

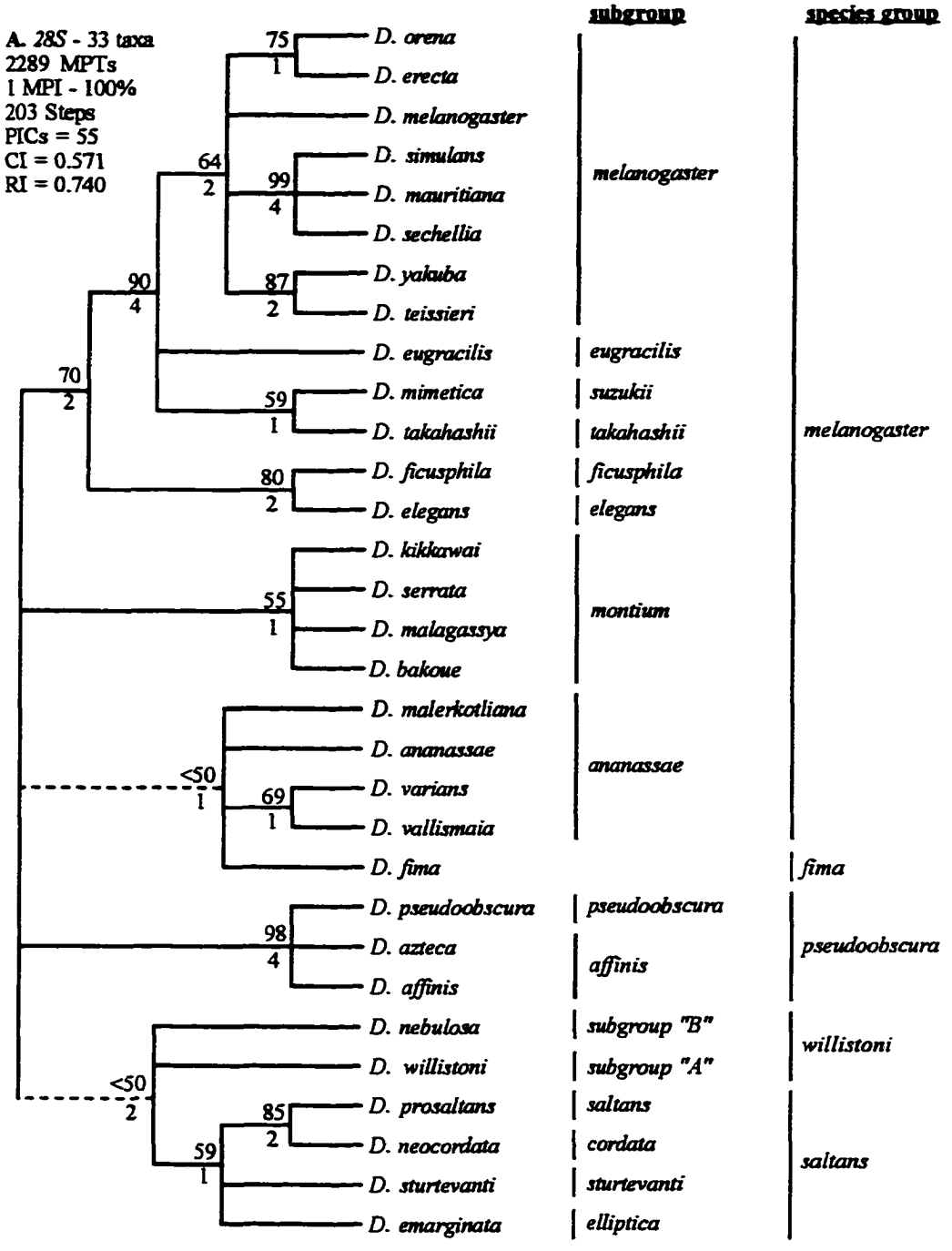

A. 28S - 33 taxa
2289 MPTs
1 MPI - 100%
203 Steps
PICs = 55
CI = 0.571
RI = 0.740
subgroup
species group
D. orena
D. erecta
D. melanogaster
D. simulans
D. mauritiana
D. sechellia
D. yakuba
D. teissieri
D. eugracilis
D. mimetica
D. takahashii
D. ficusphila
D. elegans
D. kikkawai
D. serrata
D. malagassya
D. bakoue
D. malerkotliana
D. ananassae
D. varians
D. vallismaia
D. fima
D. pseudoobscura
D. azteca
D. affinis
D. nebulosa
D. willistoni
D. prosaltans
D. neocordata
D. sturtevanti
D. emarginata
melanogaster
eugracilis
suzukii
takahashii
ficusphila
elegans
montium
ananassae
fima
pseudoobscura
affinis
subgroup "B"
subgroup "A"
saltans
cordata
sturtevanti
elliptica
melanogaster
pseudoobscura
willistoni
saltans
75
1
64
2
99
4
90
4
87
2
70
2
59
1
80
2
55
1
<50
1
69
1
98
4
<50
2
85
2
59
1

Figure 3

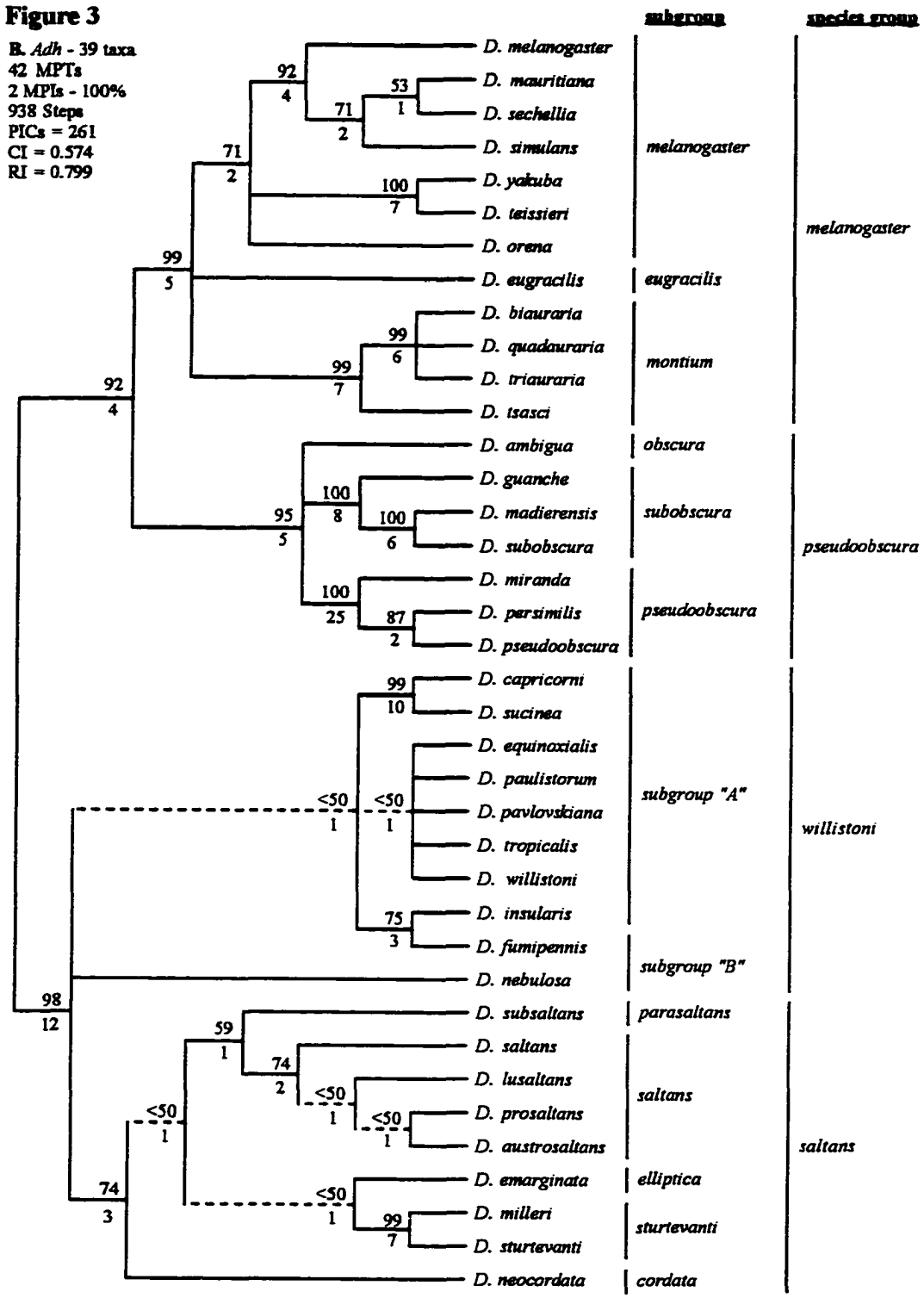

B. Adh - 39 taxa
42 MPTs
2 MPIs - 100%
938 Steps
PICs = 261
CI = 0.574
RI = 0.799
subgroup
species group
D. melanogaster
D. mauritiana
D. sechellia
D. simulans
D. yakuba
D. teissieri
D. orena
D. eugracilis
D. biauraria
D. quadauraria
D. triauraria
D. tsasci
D. ambigua
D. guanche
D. madierensis
D. subobscura
D. miranda
D. persimilis
D. pseudoobscura
D. capricorni
D. sucinea
D. equinoxialis
D. paulistorum
D. pavlovskiana
D. tropicalis
D. willistoni
D. insularis
D. fumipennis
D. nebulosa
D. subsaltans
D. saltans
D. lusaltans
D. prosaltans
D. austrosaltans
D. emarginata
D. milleri
D. sturtevanti
D. neocordata
melanogaster
eugracilis
montium
obscura
subobscura
pseudoobscura
subgroup "A"
subgroup "B"
parasaltans
saltans
elliptica
sturtevanti
cordata
melanogaster
pseudoobscura
willistoni
saltans

Figure 3

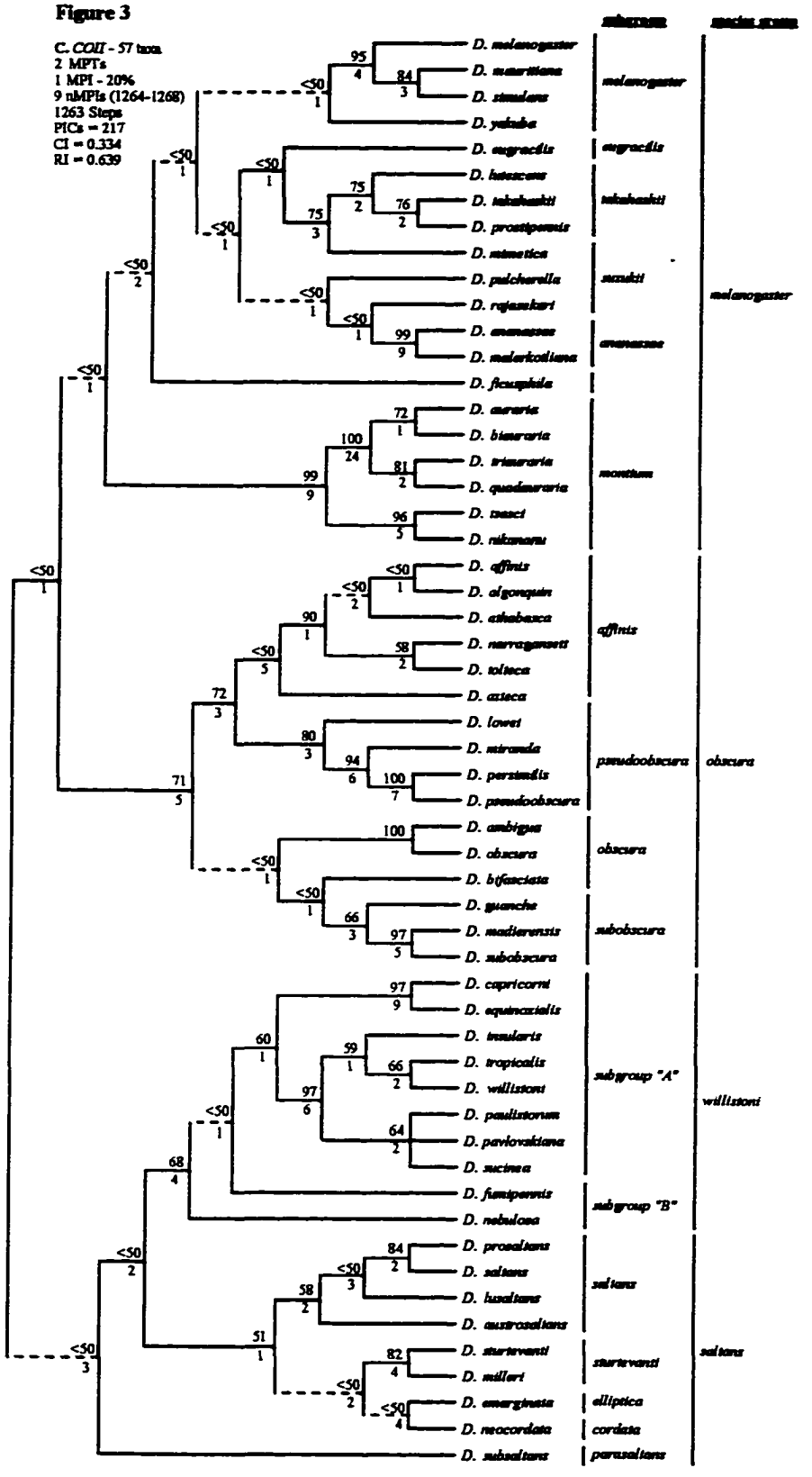
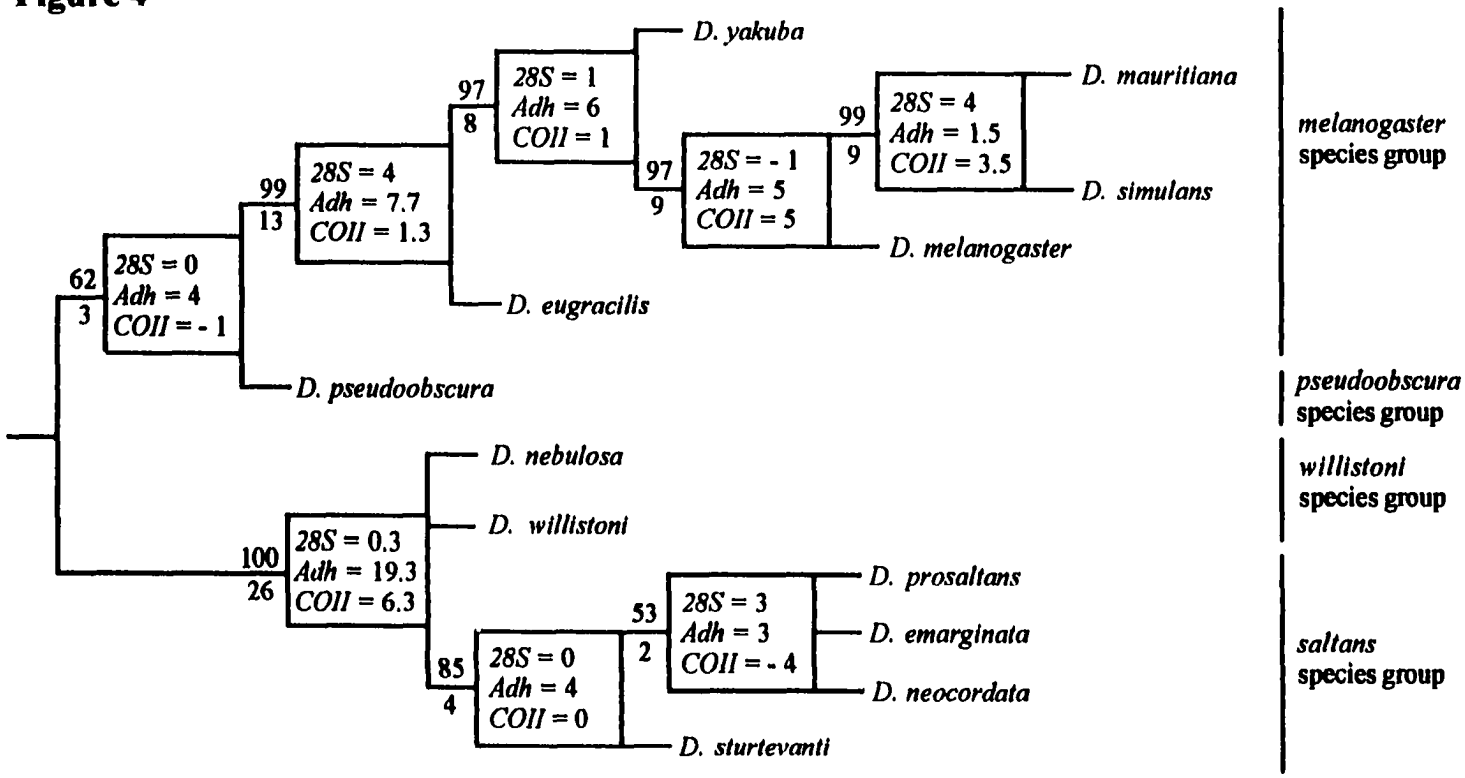
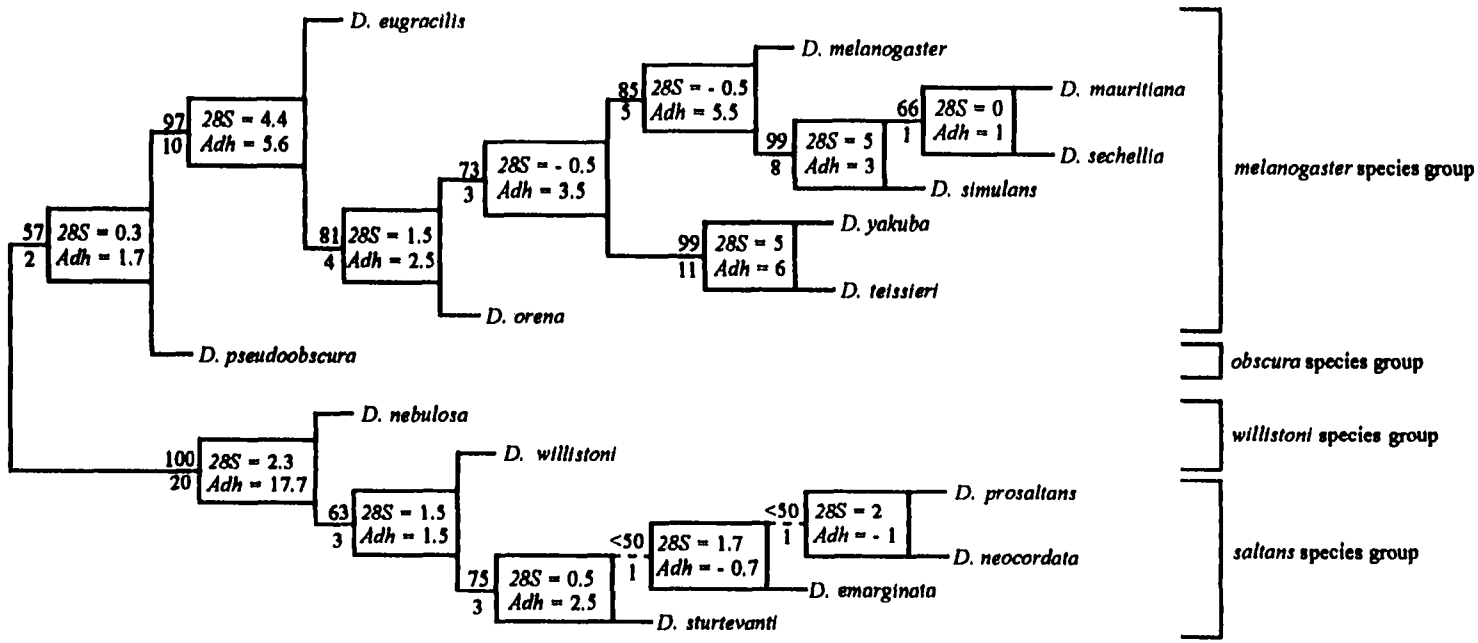

C. COII - 57 taxa
2 MPTs
1 MPI - 20%
9 nMPIs (1264-1268)
1263 Steps
PICs = 217
CI = 0.334
RI = 0.639
D. melanogaster
D. mauritiana
D. simulans
D. yakuba
D. engrectis
D. lutescens
D. takahashii
D. prostipennis
D. mimetica
D. pulcherella
D. rajasekari
D. ananassae
D. malerkotliana
D. ficusphila
D. auraria
D. bizonaria
D. trimaculata
D. quadrimaculata
D. tsacasi
D. nikananu
D. affinis
D. algonquin
D. athabasca
D. narragansett
D. tolteca
D. azteca
D. lowei
D. miranda
D. persimilis
D. pseudoobscura
D. ambigua
D. obscura
D. bifasciata
D. guanche
D. madeirensis
D. subobscura
D. capricorni
D. equinoxialis
D. innubila
D. tropicalis
D. willistoni
D. paulistorum
D. paraviakiana
D. sucinea
D. fumipennis
D. nebulosa
D. prosaltans
D. saltans
D. lusaltans
D. austrosaltans
D. sturtevanti
D. millerl
D. emarginata
D. neocordata
D. subsaltans
melanogaster
engrectis
takahashii
suzukii
ananassae
montium
affinis
pseudoobscura
obscura
subobscura
subgroup "A"
subgroup "B"
saltans
sturtevanti
elliptica
cordata
parasaltans
willistoni
saltans

Figure 4



Simultaneous Analysis - 13 taxa
2 MPTs, 1028 Steps, PICs = 322
CI = 0.687, RI = 0.632

Figure 5



A. *28S* & *Adh* - 16 taxa
1 MPT; 638 Steps
PICs = 211
CI = 0.737; RI = 0.75(

Figure 5

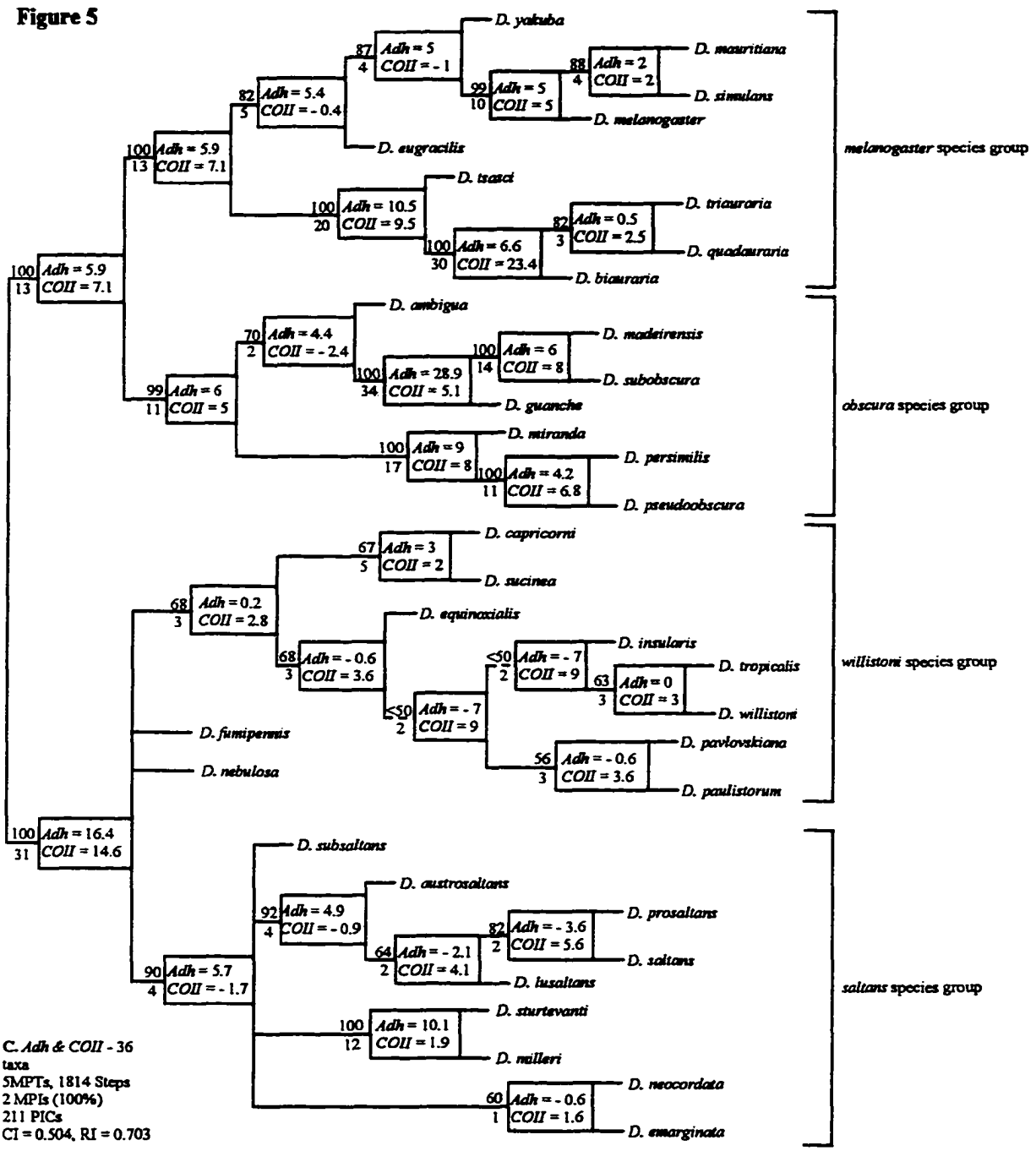
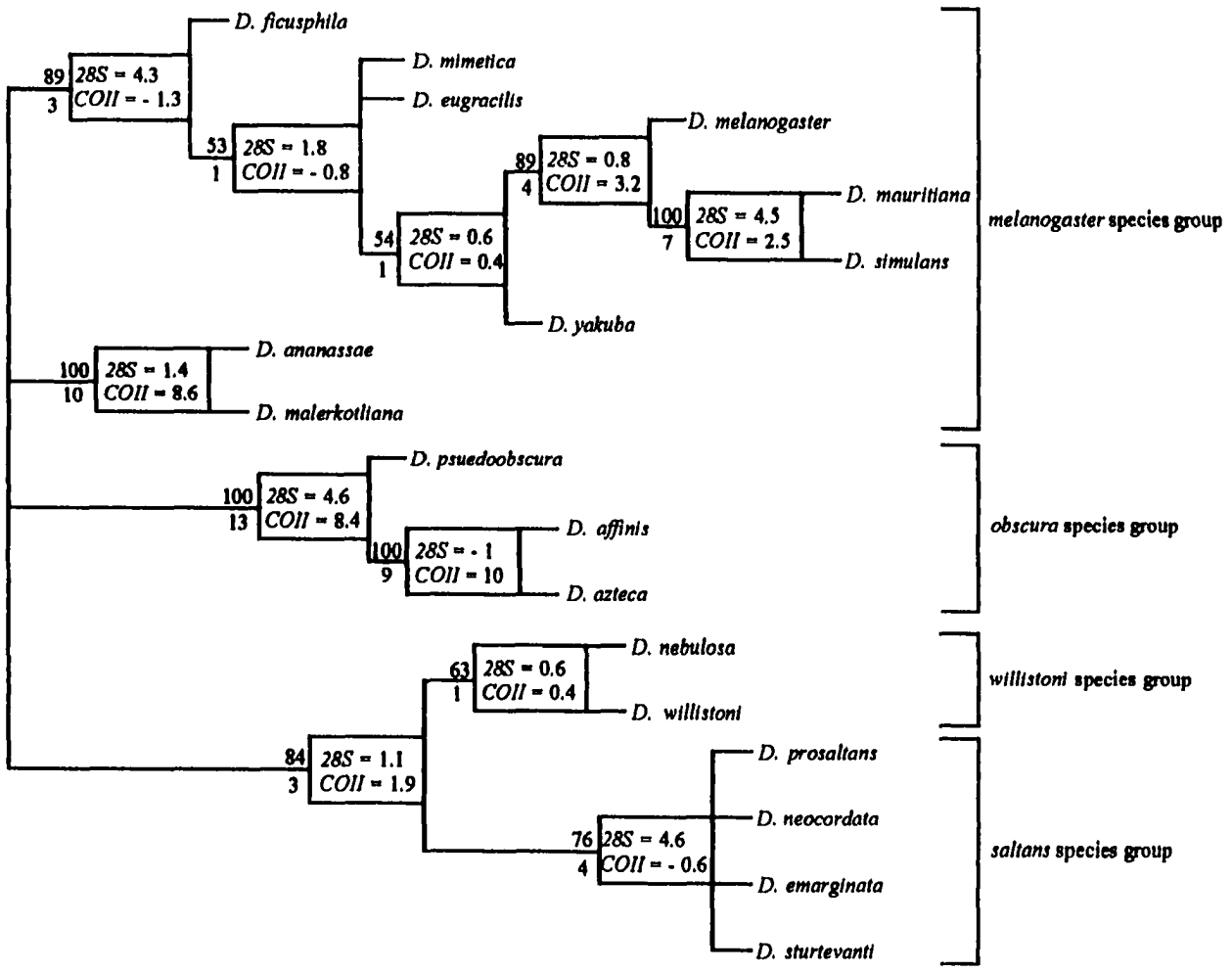
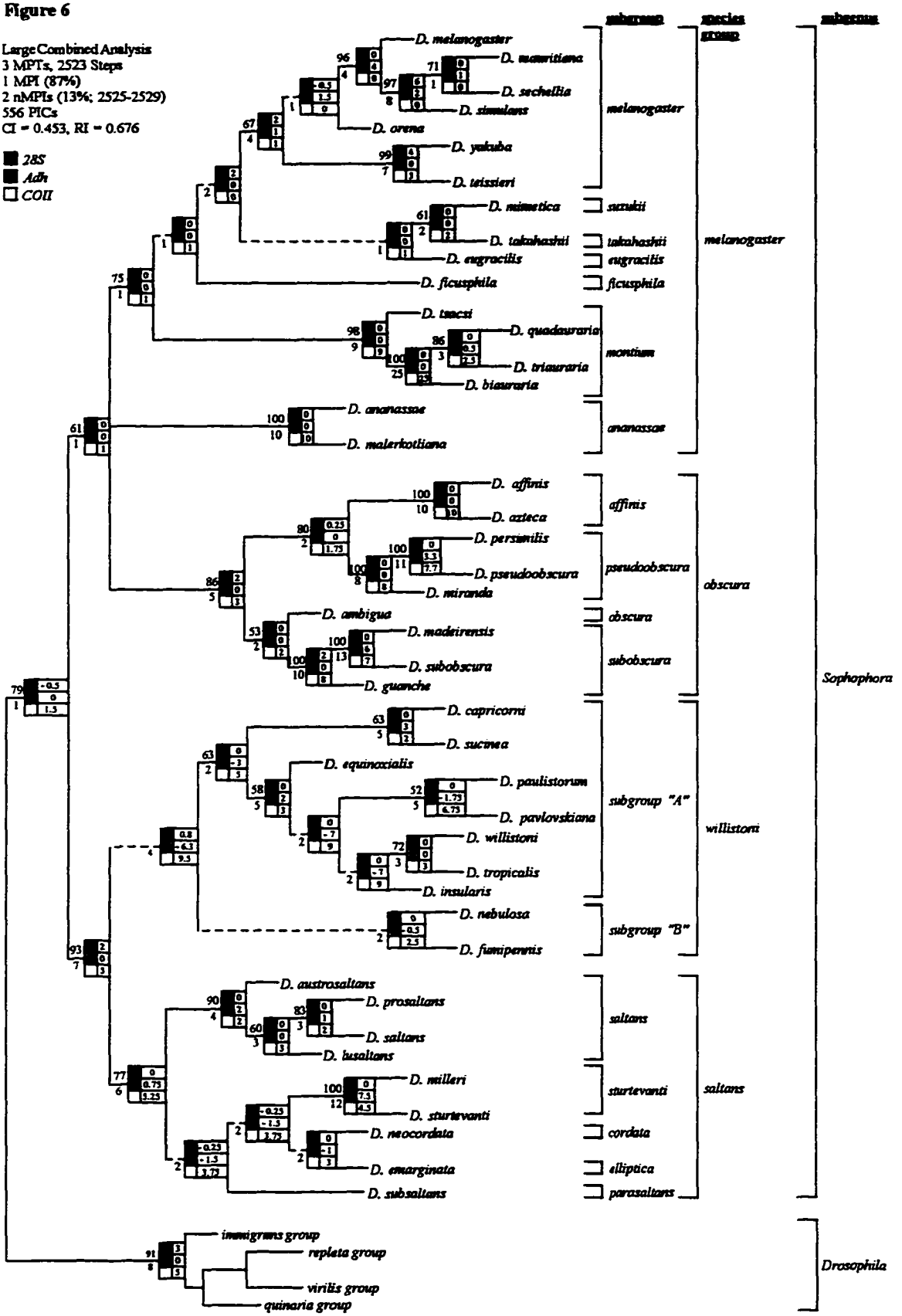

D. yakuba
87 Adh = 5 COII = - 1
4
D. mauritiana
88 Adh = 2 COII = 2
4
D. simulans
99 Adh = 5 COII = 5
10
D. melanogaster
82 Adh = 5.4 COII = - 0.4
5
D. eugracilis
100 Adh = 5.9 COII = 7.1
13
D. tsacasi
100 Adh = 10.5 COII = 9.5
20
D. triauraria
82 Adh = 0.5 COII = 2.5
3
D. quadrauraria
100 Adh = 6.6 COII = 23.4
30
D. biauraria
melanogaster species group
100 Adh = 5.9 COII = 7.1
13
D. ambigua
70 Adh = 4.4 COII = - 2.4
2
D. madeirensis
100 Adh = 6 COII = 8
14
D. subobscura
100 Adh = 28.9 COII = 5.1
34
D. guanche
99 Adh = 6 COII = 5
11
D. miranda
100 Adh = 9 COII = 8
17
D. persimilis
100 Adh = 4.2 COII = 6.8
11
D. pseudoobscura
obscura species group
D. capricorni
67 Adh = 3 COII = 2
5
D. sucinea
68 Adh = 0.2 COII = 2.8
3
D. equinoxialis
68 Adh = - 0.6 COII = 3.6
3
D. insularis
<50 Adh = - 7 COII = 9
2
D. tropicalis
63 Adh = 0 COII = 3
3
D. willistoni
<50 Adh = - 7 COII = 9
2
D. fumipennis
D. pavlovskiana
56 Adh = - 0.6 COII = 3.6
3
D. nebulosa
D. paulistorum
willistoni species group
100 Adh = 16.4 COII = 14.6
31
D. subsaltans
D. austrosaltans
92 Adh = 4.9 COII = - 0.9
4
D. prosaltans
82 Adh = - 3.6 COII = 5.6
2
64 Adh = - 2.1 COII = 4.1
2
D. saltans
D. lusaltans
90 Adh = 5.7 COII = - 1.7
4
D. sturtevanti
100 Adh = 10.1 COII = 1.9
12
D. milleri
D. neocordata
60 Adh = - 0.6 COII = 1.6
1
D. emarginata
saltans species group
C. Adh & COII - 36 taxa
5MPTs, 1814 Steps
2 MPIs (100%)
211 PICs
CI = 0.504, RI = 0.703

Figure 5



B. *28S* & *COII* - 20 taxa
10 MPTs, 770 Steps
PICs = 199
CI = 0.518, RI = 0.532

Figure 6


Large Combined Analysis
3 MPTs, 2523 Steps
1 MPI (87%)
2 nMPIs (13%; 2525-2529)
556 PICs
CI = 0.453, RI = 0.676
28S
Adh
COII
subgroup
species group
subgenus
D. melanogaster
D. mauritiana
D. sechellia
D. simulans
D. orena
D. yakuba
D. teissieri
melanogaster
D. mimetica
suzukii
D. takahashii
takahashii
D. eugracilis
eugracilis
melanogaster
D. ficusphila
ficusphila
D. tsacasi
D. quadraria
D. triauraria
D. biauraria
montium
D. ananassae
D. malerkotliana
ananassae
D. affinis
D. azteca
affinis
D. persimilis
D. pseudoobscura
D. miranda
pseudoobscura
D. ambigua
obscura
D. madeirensis
D. subobscura
D. guanche
subobscura
obscura
Sophophora
D. capricorni
D. sucinea
D. equinoxialis
D. paulistorum
D. pavlovskiana
subgroup "A"
D. willistoni
D. tropicalis
D. insularis
willistoni
D. nebulosa
D. fumipennis
subgroup "B"
D. austrosaltans
D. prosaltans
D. saltans
D. lusaltans
saltans
D. milleri
D. sturtevanti
sturtevanti
D. neocordata
cordata
D. emarginata
elliptica
D. subsaltans
parasaltans
saltans
immigrans group
repleta group
virilis group
quinaria group
Drosophila

APPENDIX D

PHYLOGENETIC RELATIONSHIPS OF FLIES IN THE FAMILY DROSOPHILIDAE INFERRED BY COMBINED ANALYSIS OF MORPHOLOGICAL AND MOLECULAR CHARACTERS

Phylogenetic relationships of flies in the family Drosophilidae inferred by combined analysis of morphological and molecular characters

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Running Head: Drosophilidae

Key Words: Drosophilidae, *Drosophila*, phylogeny, combined analysis

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Abstract

Nucleotide sequences from six molecular loci (*16S, 28S, Adh, COII, Gpdh*, and *Sod*) were examined in conjunction with three morphological data sets to estimate the phylogeny of the family Drosophilidae. Representatives from over forty major clades within this family were examined in both individual and combined analyses. The results indicate that the genus *Drosophila* is polyphyletic with respect to many genera in this family. The subgenus *Drosophila* is also not a monophyletic group. The Hawaiian Drosophilidae (Hawaiian *Drosophila* + Hawaiian *Scaptomyza* + *Engiscaptomyza*) form a clade which is the sister group of some, but not all, species groups in the subgenus *Drosophila*. In addition, these data suggest that the subgenus *Sophophora*, which is the sister clade of the subgenus *Drosophila* in previous analyses, is basal within the subfamily Drosophilinae and quite distinct from the remainder of the genus *Drosophila*. Based upon the clades recovered in this study, a tentative revision is proposed for the family Drosophilidae.

Introduction

The family Drosophilidae contains over 3,000 described species, distributed among over 60 genera (Wheeler 1982; 1986). Although some taxa within Drosophilidae have been widely studied with respect to their evolution (Patterson and Stone 1952; Powell 1997), ecology (Throckmorton 1975); genetics (Ashburner 1989; Lindsley and Zimm 1992) and development (Lawrence 1992), the phylogenetic relationships within this family are the subject of much debate (DeSalle and Grimaldi 1991; 1992; DeSalle 1994). Several studies have used either molecular sequence data (DeSalle 1992; Pelandakis and Solignac 1993; Russo *et al.* 1995) or morphological characters (Throckmorton 1975; Okada 1989; Grimaldi 1990) to assess relationships in Drosophilidae, but few molecular and morphological analysesagree completely with one another (reviewed in DeSalle and Grimaldi 1991; 1992). Furthermore, the relationships of major groups within the family Drosophilidae, with the possible exception of the genus *Drosophila*, are based primarily upon the original taxonomic descriptions of a limited number of type specimens, not strict phylogenetic analysis.

The most recent morphological revision of the family used cladistic methods to analyze over 200 morphological characters and erect a framework of tribes, subtribes, infratribes, genus complexes, genus groups, and genus subgroups to further refine higher level relationships within the Drosophilidae (Grimaldi 1990). However, this revision

conflicted with both traditional taxonomic (Throckmorton 1975) and molecular systematic (DeSalle 1992; Russo, *et al.* 1995) analyses at several points (Fig. 1).

The primary conflict concerns the placement of the spectacular radiation of endemic Hawaiian Drosophilidae (Carson 1976). This radiation is divided into two large clades, Hawaiian *Drosophila* and Hawaiian *Scaptomyza* (Throckmorton 1966; 1975; Carson 1976). Traditionally, these two clades formed a monophyletic group nested within the genus *Drosophila* (Throckmorton 1966; 1975; Carson 1976). In Grimaldi's (1990) revision, however, the Hawaiian *Drosophila*, or "drosophiloids" (Kaneshiro 1974; 1976; Throckmorton 1966; 1975), form a clade separate from what is traditionally recognized as the genus *Drosophila*. Grimaldi (1990) renamed this clade *Idiomyia* (Grimshaw 1901, *sensu* Grimaldi 1990) and placed them in the *Hirtodrosophila* genus complex, sister to the *Zygothrica* genus group, which contains the mycophageous genera *Hirtodrosophila*, *Mycodrosophila*, *Zygothrica*, *Paramycodrosophila*, and *Paraliodrosophila* (Fig. 1). The Hawaiian *Scaptomyza*, or "scaptoids" (Kaneshiro 1974; 1976; Throckmorton 1966; 1975), are not the sister group of the Hawaiian *Drosophila*, as suggested by other data, but form a clade distinct from the other Hawaiian taxa in Grimaldi's (1990) phylogeny (Fig. 1).

Grimaldi (1990) further reorganized the genus *Drosophila* by removing several subgenera and elevating them to generic rank. Two subgenera, *Scaptodrosophila* (Duda 1923) and *Hirtodrosophila* (Duda 1923), were reclassified as basal genera within the

subfamily Drosophilinae, rather than being placed within the more derived genus *Drosophila* (Fig. 1). This placement is congruent with most recent molecular data (DeSalle 1992; Russo, *et al.* 1995; however see Remsen and DeSalle 1998 for a conflicting view).

The present study utilizes an expanded species sampling strategy designed to make the molecular analyses, which have included fewer taxa, more comparable with respect to species sampling to larger morphological studies. Six molecular loci and three morphological data sets were examined in individual and combined analyses to infer phylogenetic relationships within the family Drosophilidae. The resulting improved phylogenetic hypothesis for Drosophilidae will be a powerful tool which can be used as a framework to analyze the evolutionary, genetic, developmental, and ecological information available for this clade (Powell and DeSalle 1995).

Materials and Methods

DNA Sources, Isolation, and Sequencing

Table 1 indicates the source of all specimens used in this study and the collection location, when appropriate. Live *Drosophila* stocks of some species were obtained from the National *Drosophila* Species Resource Center in Bowling Green, Ohio. Other stocks were either obtained from other *Drosophila* systematists or collected by the author. Collection permits for Costa Rica were issued by the Instituto Nacional de Biodiversidad

(InBio) through the Organizacion para Estudios Tropicales (OET). Collection permits from Ecuador were issued by (INEFAN) through collaboration with the Department of Biology at PUCE. The collection localities and methods for the species sampled in this study are presented, along with the Genbank accession numbers for each, in Appendix1.

Genomic DNA was isolated following the method of Gloor and Engels (1992). The *Adh* and *COII* loci were amplified from each taxon using standard PCR cycling conditions and oligonucleotides as described in O'Grady, Clark and Kidwell (1998). PCR products from the *Adh* gene were cloned into the TA cloning vector (Invitrogen). Two single colonies were selected and sequenced using a dsDNA cycle sequencing procedure (GIBCO-BRL). When possible, both DNA strands were sequenced from multiple clones to reduce the effect of polymerase error on the analysis. PCR products from the *COII* gene were purified by membrane filtration (Millipore) and sequenced directly using a standard dsDNA cycle sequencing protocol (GIBCO-BRL). Approximately 90% of both DNA strands were sequenced from each PCR product. The *16S* locus was amplified using primers described in DeSalle (1992). The sequences were then purified using PCR purification columns (Qiagen). Both strands were sequenced using an ABI 377 automated sequencer.

Taxon Sampling - Ingroup

Sampling at the higher level was designed to sample as many drosophilid tribes, subtribes, and genus groups, based on Grimaldi's (1990) analyses, as possible. Grimaldi's higher-level scheme was considered because his extensivly sampled phylogenetic analysis is significantly different from traditional taxonomy (Wheeler 1986) and previous molecular studies (Thomas and Hunt 1991; DeSalle 1991; reviewed in DeSalle and Grimaldi 1991; 1992). Only three major clades in his tree were not included in this study, the tribe cladochaetini, the subtribe Colocasiomynia (tribe Drosophilini) and the Dicladochaeta genus group. (Table 1). These are very small clades, which are difficult to obtain and only account for a approximately 200 of the over 3000 described species in Drosophilidae (Wheeler 1982; 1986).

Sampling within the genus *Drosophila* is critical to this analysis, as this clade contains approximately 57% of the species currently described in the family Drosophilidae (Wheeler 1986). Although this analysis only contains representatives from half of the species groups traditionally placed in the genus *Drosophila*, all of Throckmorton's (1975) major radiations within this genus are represented by multiple species groups (Appendix 1). This gives a good notion of what the relationships among major lineages within the genus *Drosophila*, as well as within the subgenus *Drosophila*.

Outgroup Sampling and Rooting

Outgroup sequences determined in the present study were chosen to include families close to Drosophlilidae. The studies of McAlpine (1989) and Grimaldi (1990) were used to select taxa. The *COII* sequences are rooted with a chloropid and three ephydrids (see Appendix 1). Outgroups not determined by this study were included as well. Following DeSalle (1992), the *16S* data set is rooted using the sequences from the mosquito *Aedes albopictus*. Although this is a very divergent sequence from *Drosophila*, it is not a random outgroup based on the criteria ofWheeler 1991 (in DeSalle 1992). The *28S* study used the diastatid fly *Delia radicum* as an outgroup (Pelendakis, *et al.* 1991). The Gpdh and Sod studies both used the genus Ceratitis to root their trees (Kwiatowski, *et al.* 1993; 1997). All individual analyses with these loci, as well as combined analyses which contain these loci, are rooted using these outgroups. The morphological analyses and the *Adh* study contain no outgroups. All individual *Adh* trees in this study are effectively unrooted, although the root was drawn using *Scaptodrosophila*, based upon previous studies (Grimaldi 1990; DeSalle 1992). All morphological trees in this study are also unrooted, but are drawn withthe subfamily Steganinae and the subfamily Drosophilinae being sister taxa.

Morphological Data

The morphological data sets of Throckmorton (1975), Okada (1989) and Grimaldi (1990) were obtained from Grimaldi (1990). Individual analyses of each morphological

data set was done using a variety of taxon sampling schemes (data not shown), but only the combined analyses of all three sets of morphological characters are shown here. This is because the data sets of Throckmorton (1975) and Okada (1989), which contain 18 and 14 characters respectively, were highly unresolved for large numbers of taxa. Appendix 1 includes the genera and species groups sampled, the representative species employed, and the number of characters included by each morphological data set.

Sequence Alignment

Alignment of the *28S* locus was exactly as in Pelendakis, *et al.* (1991) and Pelendakis and Solignac (1993). The *Gpdh* and *Sod* alignments were as in Kwiatowski, *et al.* (1997) and Kwiatowski, *et al.* (1994), respectively. The alignment of the *COII* and *Adh* coding regions was trivial. One gap, following nucleotide number 685 at the 3' end of the *COII* gene was required to align the *melanogaster* species group to the other taxa in this study. Intron regions of the *Adh*, *Gpdh* and *Sod* genes were excluded from all analyses. The *16S* sequences were aligned by eye and required several small gaps. All gaps were treated as missing data. Alignments for all loci are available from the author.

Phylogenetic Analyses -- Maximum Parsimony, Individual

Individual analyses were performed using several different taxon inclusion strategies to contrast the effect of increasing amounts of missing characters and increased

taxon sampling (Table 2). Individual data sets were analyzed with maximum parsimony (MP) and maximum likelihood (ML) as implemented in PAUP* 4.0d63 (Swofford 1998). For MP searches of the molecular data sets, several weighting schemes were tried (unweighted, transversions 2X over transitions, transversions 4X over transitions, transversions only, amino acids). The topology of each analysis was not significantly affected by weighting, so only unweighted analyses are shown here.

All MP searches in analyses A and B were performed with a branch and bound algorithm (Table 2). The genus *Scaptodrosophila* was used as an outgroup for analysis A following the results of Grimaldi (1990). Analyses B-C all employed outgroups outside the family Drosophilidae. The strength of support for each node was assessed using bootstrap proportions (BPs; Felsenstein 1985; 1988) and decay indices (DIs; Bremer 1988). Five hundred BPs were performed using the following strategy: search type = heuristic, addition sequence = random, branch swapping = TBR. MP searches in analyses C and D were performed using a heuristic search algorithm (addition sequence = random, number of replicates = 500, branch swapping = TBR). Support for nodes was determined as above.

Phylogenetic Analyses -- Maximum Likelihood, Individual

Maximum likelihood analysis, using same taxon inclusion strategies in analyses A, B, and C, will also be performed on the individual loci in this study. Points such as

computational time and model complexity must be considered when selecting a model of evolution for a given locus. A more complex model of evolution, which may estimate several parameters from the data as it infers phylogenetic relationships will be much more computationally intense than a simpler model (Felsenstein 1983). It will also tend to have a higher variance than a simpler model (Rzhetsky and Nei 1995). However, a more complex, parameter-rich model will also fit the data better (Felsenstein 1983; Goldman 1993).

A preliminary analysis was performed using the *COII* locus to determine which model of evolution most closely fit the pattern of evolution of this gene. Searches were performed in PAUP 4.0 (Swofford 1998) which estimated a variety of parameters at the same time as the algorithm estimated the phylogeny. The following searches were performed: (1) default settings: base frequency = empirical; proportion invariant sites = 0; equal rate of substitution for all sites; transition(ti)/transversion(tv) = empirical (2) base frequency = empirical; proportion invariant sites = 0; equal rate of substitution for all sites; estimate ti/tv ratio from the data, (3) proportion invariant sites = 0; equal rate of substitution for all sites; base frequencies and ti/tv ratio estimated from the data, (4) equal rate of substitution for all sites; base frequencies, ti/tv ratio, and proportion invariant sites estimated from the data, (5) rate of substitution follows a gamma shape parameter; base frequencies, ti/tv ratio, and proportion invariant sites estimated from the data, and (6) general time reversible model, base frequencies, proportion of invariant sites and gamma

shape parameter estimated from the data. All ML searches in analysis A were perfomed as follows: search = heuristic; number of replicates = 5; starting trees obtained via random stepwise addition, branch swapping = TBR.

Based upon the results of analysis A (see below), three parameters will be examined further in the larger analyses. Because of larger numbers of taxa present and the computing time commitment required, the ti/tv ratio, proportion of invariant sites, and a gamma shape substitution parameter will be estimated on MP trees in analyses C and D. Once obtained, these parameter estimates will be incorporated into a ML analysis to infer the phylogenetic relationships of taxa for the loci in this study (Sullivan, *et al.* 1995, but see Gu and Zhang 1997). ML searches in analyses B and C will be executed as in analysis A.

Phylogenetic Analyses -- Combined

Only MP was used to analyze combined data partitions. Search strategies for combined analyses were identical to individual analyses A-C. The 250 morphological characters analyzed here (Throckmorton 1975; Okada 1989; Grimaldi 1990) were combined and analyzed using maximum parsimony. All morphological characters were treated as unordered and unweighted.

Partitioned Bremer support (Baker and DeSalle 1997) was also performed in combined analyses to measure the amount of support provided by each individual

partition to the DI for every node in the combined analysis phylogenies (Figs. 3, 5, and 7). Partitioned Bremer support (PBS) shows the contribution of each partition to the decay index of every node on the total evidence tree. To obtain the PBS value for a given node on the total evidence tree, the length of the partition on the unconstrained total evidence tree was subtracted from the length of a partition on a tree constrained to not contain the node of interest. If the partition supports a relationship represented by a node in the total evidence tree, then the PBS value will be positive. If, on the other hand, a partition supports an alternative relationship, the PBS value will be negative, indicating incongruence with the simultaneous analysis. The magnitude of PBS values indicate the level of support for, or incongruence with, a node. The sum of all partition lengths for any given node will always equal the decay index for that node on the total evidence tree. Using this method allowed determination of the relative contribution of each partition to the different simultaneous analysis trees (Figs. 3, 5, and 7).

Results

Analysis A

Figure 2 shows the results of individual analyses of clades with representatives from all seven partitions (Analysis A). Bootstrap support for clades in these analyses is, for the most part, weak. The *obscura-melanogaster* clade is supported in the *Adh*, *COII*, *Gpdh*, and *Sod* analyses. The *16S* and *28S* loci support the sister group relationship of

the *obscura* and *willistoni* groups, a result which has not been seen in previous analyses. The subgenus *Sophophora* is only monophyletic in one analysis, *16S*. No individual analysis indicates that the subgenus *Sophophora* is the sister clade of the subgenus *Drosophila*. Instead, several analyses (*28S*, *Gpdh*, and *Sod*) indicate that the genus *Zaprionus* may be the sister group of the *repleta* group, the subgenus *Drosophila* representative in this analysis.

Figure 3 shows the results of combined analyses for these taxa. The morphological partition (Fig. 3A) is mostly unresolved, although it is able to resolve the *obscura-melanogaster* relationship. The molecular partition is, by contrast, more resolved (Fig. 3B). The combined analysis of six loci resolves a monophyletic *Sophophora*, although relationships within this clade are unconventional, and a *repleta-Zaprionus* clade with high support (Fig 3B; BP=92%, DI=15). Dividing the molecular partition into nuclear and mitochondrial loci, we see that support for the *repleta-Zaprionus* clade comes mostly from the nuclear partition (Fig. 3C), although PBS values in the combined analysis of all seven partitions shows that all loci except for *16S* support this clade (Fig. 3E). A monophyletic *Sophophora* is supported by the mitochondrial partition and the 28S locus (Figs. 3D-E). The unconventional *obscura-willistoni* clade is primarily due to the influence of the *16S* locus (PBS = 27) and should not be considered strongly supported because most other loci conflict with this node.

Results of Preliminary Likelihood Analysis A

As more complicated models of evolution were employed for the COII locus in analysis A, the likelihood score decreased markedly (from 2268.1964 in model 1 to 2123.9568 in model 6), indicating that the likelihood model was fitting the pattern of evolution of the sequence. However, with the exception of branch length differences, the relationships of taxa in the tree did not change. The topology of all *COII* likelihood trees was identical to the *COII* MP phylogeny. The *COII* study showed that the greatest decrease in likelihood score was when the proportion of invariant sites was added (2240.0148 in model 3 to 2164.6954 in model 4) and the addition of a GTR model (2164.1863 in model 5 to 2123.9568 in model 6). The effect of estimating a gamma shape parameter, independent of estimating the proportion of invariant sites was not assessed specifically, but the decrease is minimal with both parameters estimated (2164.6954 in model 4 vs. 2164.1863 in model 5).

Analysis B

Results of individual Analysis B searches are shown in Figs. 4A-E. Although bootstrap proportions and decay indices are low for most clades in these trees, the *Adh* and *Sod* loci give strong support for several traditional relationships (Figs. 4B, D). The subgenus *Sophophora* is monophyletic only in the *Adh* tree (Fig. 4A). Within *Sophophora*, however, the *melanogaster-obscura* lineage is supported in the *Adh*, *Gpdh*,

and *Sod* trees (Figs. 4B-D) and the *saltans-willistoni* clade is recovered in the *Adh* and *Sod* phylogenies (Figs. 4B, D). Interestingly, no analysis indicates that the subgenus *Drosophila* is a clade, although several analyses show that groups within this subgenus are monophyletic. A variety of genera, including *Hirtodrosophila*, *Zaprionus*, and *Scaptomyza* make this subgenus paraphyletic (Fig. 4). Furthermore, the genus *Drosophila* groups examined in these analyses (*Sophophora*, *Drosophila* and *Dorsilopha*) do not form a clade.

The results of combined analyses of these taxa is shown in figure 5. The morphological characters indicate that the subgenus *Drosophila* is monophyletic, but are unable to resolve a monophyletic subgenus *Sophophora* or genus *Drosophila* (Fig. 5A). Furthermore, these characters indicate that the genus *Zaprionus* is more closely related to the subgenus *Drosophila* than the other subgenera in the genus *Drosophila* (Fig. 5A). The molecular partitions indicate that the subgenus *Sophophora*, and that the *melanogaster-obscura* and *saltans-willistoni* clades within this group, is monophyletic (Fig. 5B). The genus and subgenus *Drosophila* are, however, paraphyletic with respect to the genera *Zaprionus*, *Scaptomyza*, and *Hirtodrosophila* (Fig 5B). Figure 5C shows the results of combined analysis of all nu partitions in this study. The subgenus Sophophora and the lineages within it are all monophyletic. Again, the subgenus *Drosophila* is not a clade, *Scaptomyza* is closely related to the *virilis-repleta* lineage and *Zaprionus* is the sister taxon of the *immigrans-quinaria* lineage (Fig. 5C). However, in contrast to other

analyses in this study, *Hirtodrosophila* is basal to the genus *Drosophila*, not within it. The results of combined analysis of the mt partitions are shown in figure. 5D. These loci are unable to resolve relationships among the taxa as well as the nu loci. The subgenera *Sophophora* and *Drosophila*, as well as the genus *Drosophila*, as not monophyletic. The combined analysis of all data is shown in figure 5E. This analysis indicates that the subgenera *Drosophila* and *Sophophora* are clades. However, relationships among the three *Drosophila* lineages analyzed here and the genera *Zaprionus*, *Scaptomyza* and *Hirtodrosophila* are unresolved. *Chymomyza* and *Scaptodrosophila* are clearly basal to this large clade (Fig. 5E).

Analysis C

This analysis expands taxon sampling further to include about forty major clades within the family Drosophilidae. Individual analyses are shown in figures 6A-D. Analyses for the *Gpdh* and *Sod* loci are not shown because they are identical to those in analysis B. Figure 6A shows the results of the individual analysis of the *28S* locus. Support for relationships in this phylogeny, with the exception of the *virilis-polychaeta* lineage, is weak. The subgenus *Sophophora* is unresolved in this analysis. Some relationships within the subgenus *Drosophila* are resolved, but the genus *Samoaia* also falls within this group, making the subgenus paraphyletic (Fig. 6A). The *Adh* locus (Fig. 6B) is, by comparison, much more resolved. The subgenus *Sophophora* forms a well

supported clade. The Hawaiian *Drosophila* are monophyletic and the sister clade of the *Scaptomyza-Engiscaptomyza* clade, although the genus *Scaptomyza* seems to be paraphyletic with respect to *Engiscaptomyza*. The Hawaiian Drosophilidae (Hawaiian *Drosophila* + *Engiscaptomyza* + *Tantalia*) are also paraphyletic with respect to the continental species of *Scaptomyza*. The subgenus *Drosophila* is, again, not monophyletic. The *virilis-repleta* lineage is the sister group of the Hawaiian *Drosophila-Engiscaptomyza-Scaptomyza* clade and the *immigrans* lineage is basal to all these species, making the subgenus *Drosophila* paraphyletic.

The results of individual analyses of the mt *16S* and *COII* loci are shown in figures 6C and D, respectively. These phylogenies are both poorly resolved and display little support, in terms of bootstrap proportions or decay indices, for most clades. Some relationships are, however, well supported (Figs. 6C,D).

Figure 7 shows a combined analysis of all taxa which are present in three or more of the data partitions in this study. PBS values were calculated for all nodes in the tree, but are only shown to highlight selected relationships. The results of this analysis indicate that the Hawaiian *Drosophila*, Hawaiian *Scaptomyza* and Continental *Scaptomyza* form a clade. This Hawaiian *Drosophila* + *Scaptomyza* clade is part of a larger clade which includes some, but not all, species groups traditionally placed in the subgenus *Drosophila*. Although this node is unresolved, flies placed in the *virilis-repleta* radiation (Throckmorton 1975), the *funebris-immigrans-melanica* clade, or the *bromeliae*

species group are possible sister groups of the Hawaiian radiation. The *Hirtodrosophila* Genus Complex (*sensu*, Grimaldi 1990) is also a well supported clade which is the sister group of the remaining species groups in the subgenus *Drosophila*, along with the subgenera *Phloridosa* and *Siphlodora*. Interestingly, these groups were all placed in the *tripunctata* radiation by Throckmorton (1975). Finally, the subgenus *Sophophora* is basal to the other clades in the genus *Drosophila*, a result which has not been seen with other molecular data.

Discussion

The expanded taxon sampling employed in the current study, along with the combination of several molecular and morphological data sets sheds new light on the phylogenetic relationships of some groups in the family Drosophilidae. Most surprisingly, neither the genus *Drosophila* nor the subgenus *Drosophila* are monophyletic. The genus *Drosophila*, as currently defined (Powell and DeSalle 1995), is not monophyletic with respect to the genera *Hirtodrosophila*, *Mycodrosophila*, *Paramycodrosophila*, *Samoaia*, *Scaptomyza*, *Zaprionus* and *Zygothrica* (Fig. 8). The subgenus *Drosophila* falls into at least two major clades which roughly correspond to the *tripunctata* and *virilis-repleta* "radiations" proposed by Throckmorton (1975) on the basis of his morphological investigations (Fig 8). A possible third clade, closely related to the *virilis-repleta* clade, consists of *D. funebris*, *D. immigrans*, and *D. melanica*. Another

significant result is that the endemic Hawaiian Drosophilidae form a clade, although this group also contains at least two continental subgenera placed in the genus *Scaptomyza* (Fig. 8).

Based on the results of these phylogenetic analyses, the family Drosophilidae can be broken into several major clades (Table 3). The *Scaptomyza* clade consists of the continental subgenera of *Scaptomyza* as well as the paraphyletic Hawaiian subgenera (Fig. 8). The Hawaiian *Drosophila* form another clade (Fig. 8). The Scaptomyza + Hawaiian Drosophila + Engiscaptomyza lineages form another clade, which can be thought of as a Hawaiian Drosophilidae clade, with the inclusion of the continental *Scaptomyza* (Fig. 8). Related to the Hawaiian Drosophilidae clade are three other lineages: the *virilis-repleta* clade, which consists of several species groups placed in the *virilis-repleta* "radiation" by Throckmorton (1975), the *funebris-immigrans-melanica*, or simply *funebris*, clade, and the *bromeliae* species group (Fig. 8). It is somewhat surprising to find *Drosophila immigrans* related to *D. funebris* and *D. melanica*, instead of to the *tripunctata* clade (Fig. 7). This may be because Old World members basal to the *tripunctata* radiation, such as *D. immigrans*, were inadequately sampled. Further sampling, within both the *virilis-repleta* and "*immigrans-Hirtodrosophila* radiation" (Throckmorton 1975) may alter this relationship as well as unite the *virilis-repleta* clade and the *funebris* clades. The Hawaiian Drosophilidae, *funebris*, *bromeliae*, and *virilis-repleta* clades collectively form

what can be thought of as the "*Drosophila* #1 clade" because it contains many of the species groups and subgenera previously placed in *Drosophila* (Fig. 8).

The "*Drosophila* #2 clade" consists of the remaining species groups traditionally placed in the genus *Drosophila* (Fig. 8). Related to this clade is the mycophagous *Hirtodrosophila* Genus Complex (Grimaldi 1990) and the genus *Samoaia*. The majority of species in the *Drosophila* #2 clade are either mycophagous or flower breeders, similar to the *Hirtodrosophila* Genus Complex species. Two other groups, the monotypic subgenus *Dorsilopha* and the genus *Zaprionus*, are clearly related to the species in the *Drosophila* #1 + *Drosophila* #2 clades. The subgenus *Sophophora* corresponds to another clade in this phylogeny, the *Sophophora* clade (Fig. 8). The genera Chymomyza and Scaptodrosophila, considered to be basal in the subfamily Drosophilidae (Throckmorton 1975; Grimaldi 1990; DeSalle 1992; Powell and DeSalle 1995), form the "basal clade" in this analysis as well. Contrary to previous results, the subfamily *Steganinae* is not monophyletic in this analysis. This could be due to inadequate sampling within the group or selection of inappropriate outgroup taxa. Several outgroups employed in this study, primarily *Ceratitis* (*Gpdh* and *Sod*) and *Aedes* (*16S*) are very distantly related to Drosophilidae. These may be "random outgroups" and are simply attaching to a long branch near the base of the tree. A monophyletic Steganinae is XX steps longer than the most parsimonious tree with this group paraphyletic. Additional

sequencing, both within Steganinae and in families close to Drosophilidae is required to resolve this issue.

The division of the family Drosophilidae into nested clades of related species is long overdue. The "multiple radiations" view of Throckmorton (1975; 1982) and the morphological cladistic analysis of Grimaldi (1990) both predict clades seen in this combined analysis. Throckmorton's view, in particular, is surprisingly accurate when tested using phylogenetic criteria. Both studies, however, are unfortunately highly unresolved when analyzed separately. Although the present study is a first step in improving the resolution of these polytomies and revising the family Drosophilidae using a combined phylogenetic approach, it is clear that additional taxa and characters are needed. Species sampling should be increased to include (1) more genera within the subfamily Steganinae and (2) more subgenera and species groups, particularly from the Old World tropics, in what Throckmorton (1975) refers to as the "*immigrans-Hirtodrosophila* radiation. Furthermore, additional molecular data, from protein coding nuclear loci, and morphological characters will be need to resolve uncertainties and increase clade support in the proposed phylogeny.

These analyses indicate that the genus *Drosophila* should be divided into at least three, and possibly more, genera. However, such a division could have wide-ranging implications for other branches of biology. For example, the formation of the genus *Sophophora* from the current subgenus *Sophophora* would invalidate the nomenclature

currently in use in genetics and developmental biology. Changing *Drosophila melanogaster* to "*Sophophora melanogaster*" would be met with widespread, and perhaps justifiable, resistance. Retaining the current taxonomic system while employing a clade-based, phylogenetic division of the family might be a more reasonable solution (De Queiroz and Gauthier 199?).

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Table 1. Ingroup Sampling - Tribal Level

Subfamily	Tribe	Subtribe	Genus Group	Genus*
Steganinae	Steganini	Steganina	ND	*Stegana*
		Leucophengina	ND	*Leucophenga*
	Gitonini	Acletoxenina	*Pseudiastata*	*Rhinoleucophenga*
		Gitonina	ND	*Gitona*
			Amiota	*Amiota*
Drosophilinae	Cladochaetini			NS
	Drosophilini	Colocasiomynia		NS
		Drosophilinia	ND	*Scaptodrosophila*
			Chymomyza	*Chymomyza*
			Zygothrica	*Zygothrica*
				Hirtodrosophila
				Mycodrosophila
				Paramycodrosophila
			Drosophila	*Drosophila*
			Styloptera	*Liodrosophila*
			Dicladochaeta	NS
			Zaprionus	*Zaprionus*
				Samoaia
			Scaptomyza	*Scaptomyza*

ND - This taxonomic level was not designated for the taxa in question.

NS - This clade was not sampled in the present study.

* - The species used to represent each genus are listed in Appendix 1.

Table 2.

Analyses Performed

Search Name	Type of Search	Partitions Examined	Sampling Strategy	Search Information
A	Individual	16S, 28S, Adh	7 partitions	MP - BandB
		COII, Gpdh, Sod	(6)	ML - Heuristic (5),
	Combined	mtDNA, nuDNA		MP - BandB
		morphology, molec		
		all data		
B	Individual	16S, 28S, Adh	5+ partitions	MP - Heuristic (500)
		COII, Gpdh, Sod	(~15)	ML - Heuristic (5),
	Combined	mtDNA, nuDNA		MP - Heuristic (500)
		morphology, molec		
		all data		
C	Individual	16S, 28S, Adh	3+ partitions	MP - Heuristic (500)
		COII, Gpdh, Sod	(~40)	
	Combined	mtDNA, nuDNA		MP - Heuristic (500)
		morphology, molec		
		all data		

Table 3. Phylogenetic Clades Defined in this Study

Proposed Phylogenetic Clade	Taxonomic Groups Included in that Clade
1. *Scaptomyza* clade	Continental and Hawaiian *Scaptomyza*
2. Hawaiian *Drosophila* clade	Hawaiian *Drosophila*
3. Hawaiian Drosophilidae clade	*Scaptomyza* and Hawaiian *Drosophila* clades + *Engiscaptomyza*
4. *funebris* clade	*funebris*, *immigrans* and *melanica* species groups
5. *virilis-repleta* clade	*drefusi*, *mesophargmatica*, *polychaeta*, *virilis* and *repleta* species groups
6. *bromeliae* clade	*bromeliae* species group
7. *Drosophila* clade #1	Hawaiian Drosophilidae, *funebris*, *bromeliae* and *virilis-repleta* clades
8. *Hirtodrosophila* Genus Complex	*Hirtodrosophila*, *Zygothrica*, *Paramycodrosophila*, and *Mycodrosophila*
9. *tripunctata* clade	*gurani*, *quinaria*, *testecea*, and *tripunctata* species groups
10. *Drosophila* clade #2	*tripunctata* clade + *Phloridosa* + *Siphlodora*
11. *Sophophora* clade	*melanogaster*, *obscura*, *saltans*, and *willistoni* species groups
12. *Steganinae* "pseudo-clade"	*Amiota*, *Gitona*, and *Rhinoleuclophenga*

Figure Legends

FIG. 1. - Comparison of phylogenetic relationships proposed by Grimaldi's (1990) morphological analysis (A) and a variety of molecular analyses (B).

FIG. 2. - Results of individual analyses of mt DNA sequences. Each phylogeny is shown with the length, in base pairs (bp) of the partition, the number of parsimony informative (PI) characters in the data set, the number of MPTs found, the number of steps on each MPT, and the ensemble consistency and retention indices (CI and RI, respectively). BPs are shown above and DIs are shown below each node.

FIG. 3A. - Results of combined analysis of morphological partitions (analysis A). Trees are unrooted, but drawn with Scaptodrosophila at the base. Bootstrap proportions are above the nodes, decay indices are below.

FIG. 3B. - Results of combined analysis of molecular partitions (analysis A). Trees are rooted with outgroups as specified in materials and methods. Bootstrap proportions are above the nodes, decay indices are below.

FIG. 3C. - Results of combined analysis of nuclear partitions (analysis A). Trees are rooted with outgroups as specified in materials and methods. Bootstrap proportions are above the nodes, decay indices are below.

FIG. 3D. - Results of combined analysis of mitochondrial partitions (analysis A). Trees are rooted with outgroups as specified in materials and methods. Bootstrap proportions are above the nodes, decay indices are below.

FIG. 3E. - Results of combined analysis of molecular and morphological partitions (analysis A). Trees are rooted with outgroups as specified in materials and methods. Bootstrap proportions are above the nodes, decay indices are below. PBS values are shown in the boxes, numbers preceeded by a black box are positive and those preceeded by a white box are negative.

FIG. 4A. - Results of combined mitochondrial analysis. Each phylogeny is shown with the length, in base pairs of the partition, the number of parsimony informative characters in the data set, the number of MPTs found, the number of steps on each MPT, and the CI and RI values. BPs (above node), Dis (below node), and PBS values (in boxes at node) are indicated. Branch lengths are not proportional to change, they vary only for clarity.

FIG. 4B. - Results of combined nuclear analysis. Each phylogeny is shown with the length, in base pairs of the partition, the number of parsimony informative characters in the data set, the number of MPTs found, the number of steps on each MPT, and the CI and RI values. BPs (above node), Dis (below node), and PBS values (in boxes at node) are indicated. Branch lengths are not proportional to change, they vary only for clarity.

FIG. 5. - Results of combined analysis #2. Each phylogeny is shown with the length, in base pairs of the partition, the number of parsimony informative characters in the data set, the number of MPTs found, the number of steps on each MPT, and the CI and RI values. BPs (above node), Dis (below node), and PBS values (in boxes at node) are indicated. Branch lengths are not proportional to change, they vary only for clarity.

FIG. 6. - Summary of phylogenetic relationships within the *Drosophila obscura* species group. (A). Phylogenetic relationships supported by individual and combined analyses in this study. (B). Phylogenetic relationships proposed by Gleason, *et al.* (1997) based on analysis of mtDNA sequences. Figure numbers of the analysis in the present study supporting each node is shown above that node. Numbers associated with named clades correspond to references which support that clade. 1. Dobzhansky (1935), 2. Sturtevant (1942), 3. Buzzati-Traverso and Scossiroli (1955), 4. Throckmorton (1975), 5.

Anderson, *et al.* 1977, 6. Lakovaara and Saura (1982), 7. Cariou, *et al.* 1988, 8. Goddard, *et al.* (1990), 9. Bachmann, *et al.* (1992), 10. Ruttkay, *et al.* (1992), 11. Beckenbach, *et al.* (1993), 12. Bachmann and Sperlich (1993), 13. Krimbas 1993, 14. Barrio, *et al.* 1994, 15. Powell and DeSalle (1995), 16. Russo, *et al.* (1995), 17. Wells (1996), 18. Barrio and Ayala (1997), 19. Gleason, *et al.* (1997), 20. This study.

Figure 1

A. *Morphological Data*
(Grimaldi 1990)

B. *Molecular Data - Composite Phylogeny*
16S (DeSalle 1992)
28S (Pelendakis, et al. 1993)
Adh (Russo, et al. 1995 and others)
Gpdh (Kwiatowski, et al. 1997)
Sod, (Kwiatowski, et al. 1994)

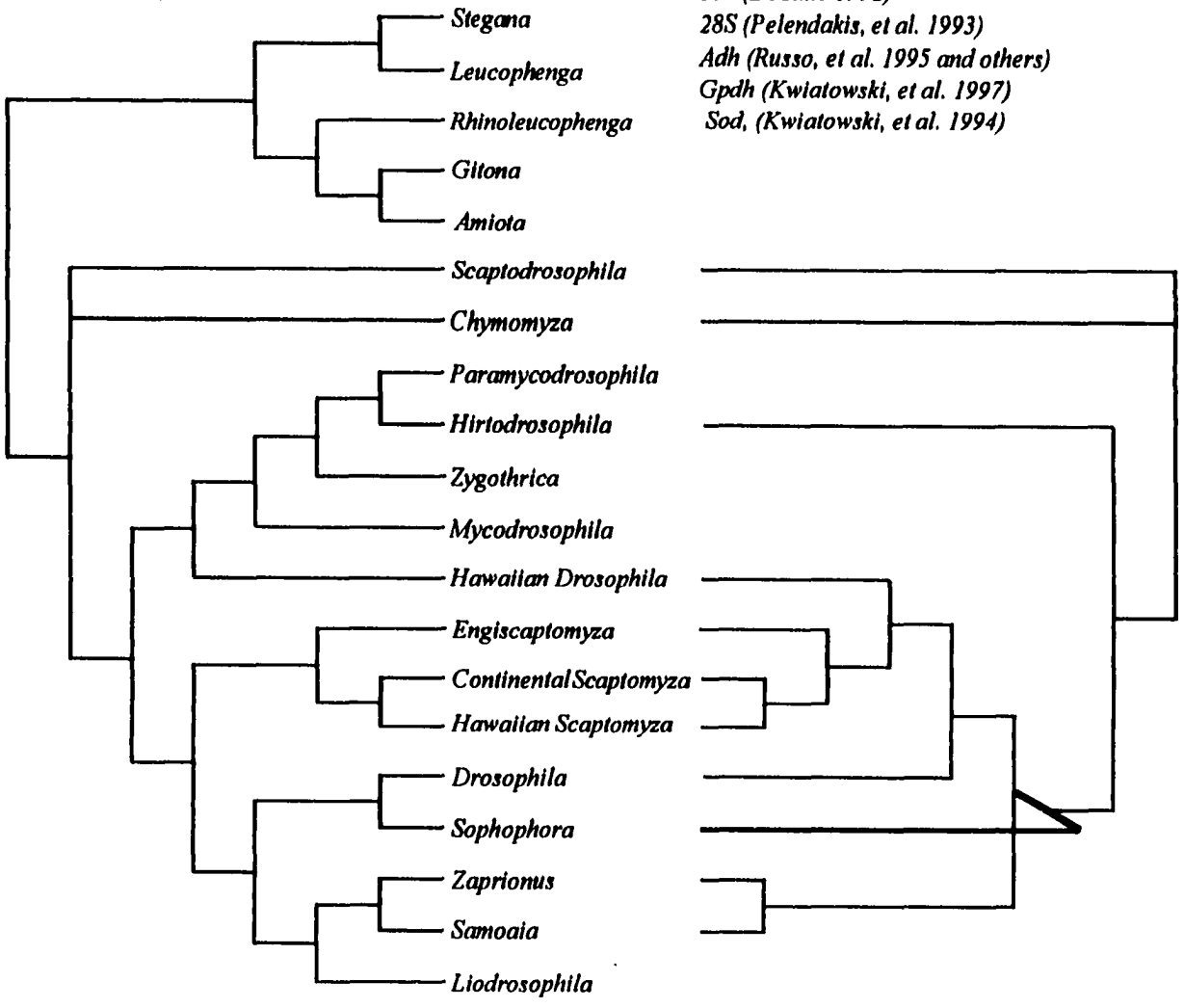
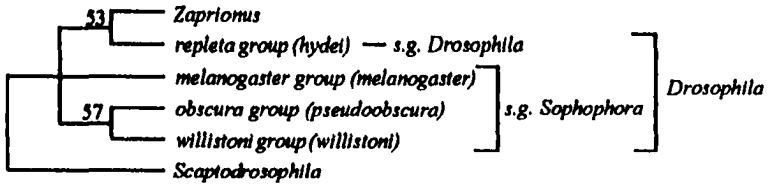
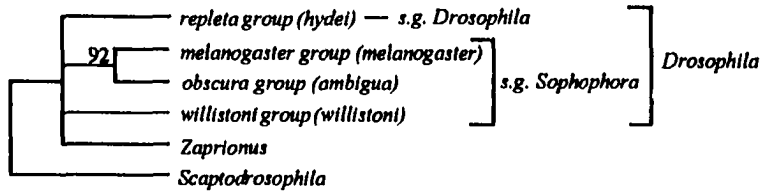


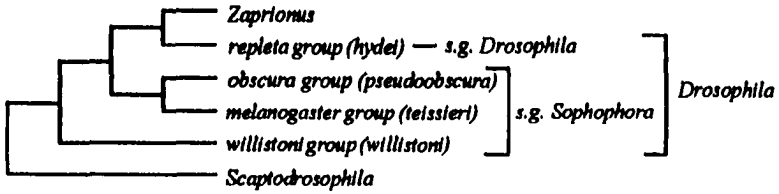
Figure 2



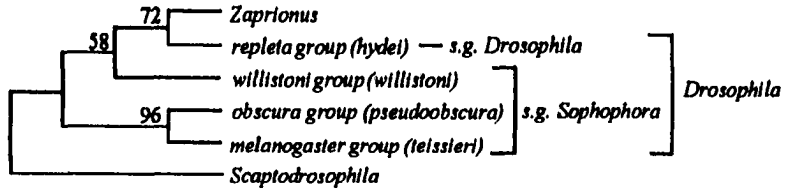
A. *28S*
2 MPTs; 89 Steps; 24 PICs
CI = 0.798; RI = 0.419



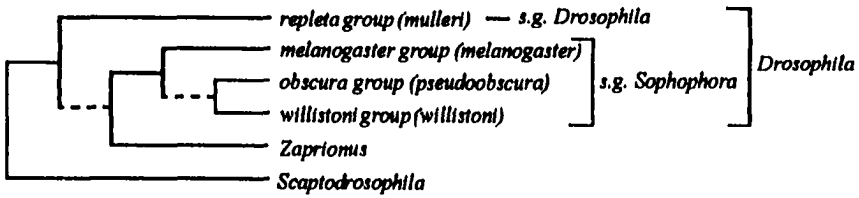
B. *Adh*
2 MPTs; 541 Steps; 145 PICs
CI = 0.786; RI = 0.366



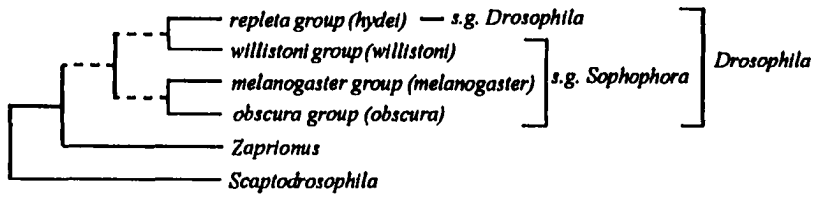
C. *Gpdh*
1 MPT; 396 Steps; 122 PICs
CI = 0.735; RI = 0.305



D. *Sod*
1 MPT; 335 Steps; 102 PICs
CI = 0.770; RI = 0.403

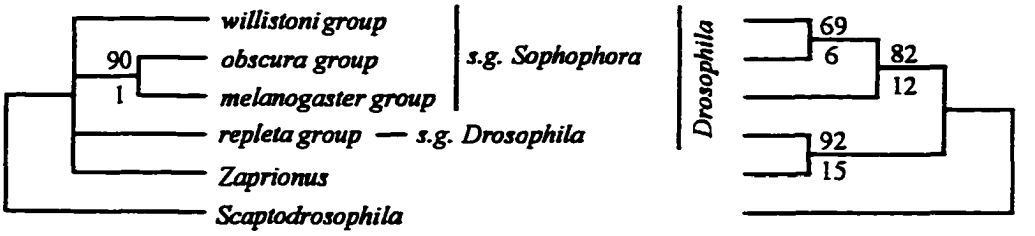


E. *16S*
1 MPT; 173 Steps; 44 PICs
CI = 0.936; RI = 0.771



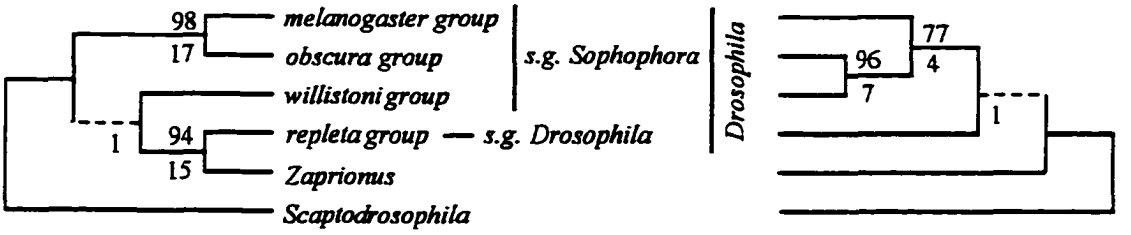
F. *COII*
1 MPT; 291 Steps; 82 PICs
CI = 0.766; RI = 0.299

Figure 3



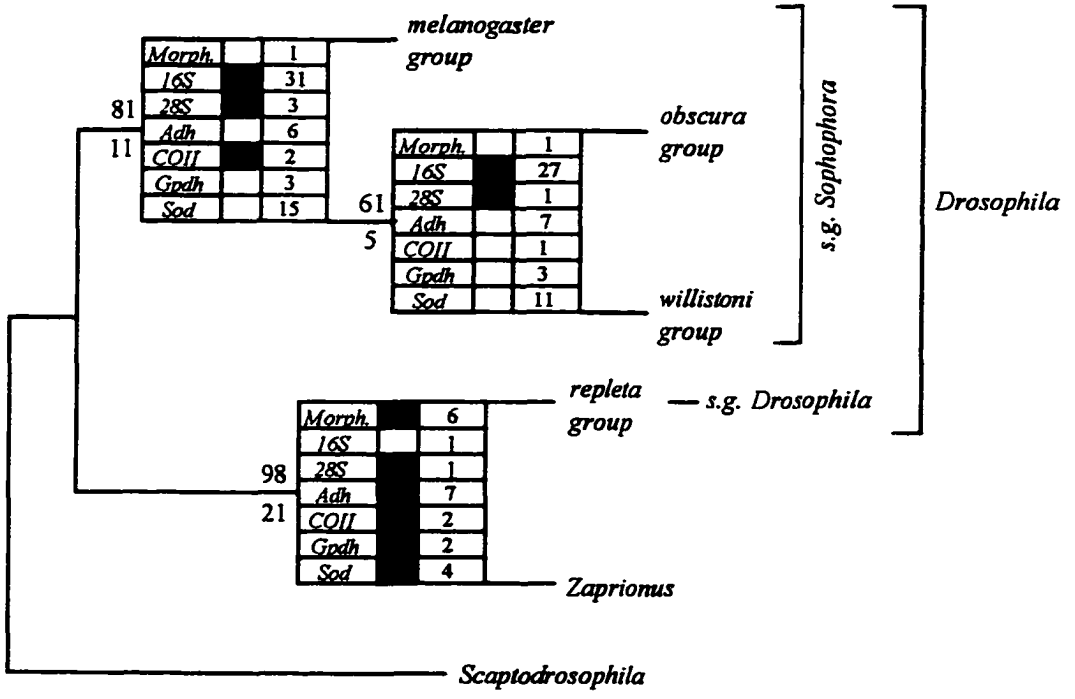
A. *Morphological Partitions*
4 MPTs; 54 Steps; 17 PICs
CI = 0.926; RI = 0.765

B. *Molecular Partitions*
1 MPT; 1859 Steps; 519 PICs
CI = 0.769; RI = 0.326



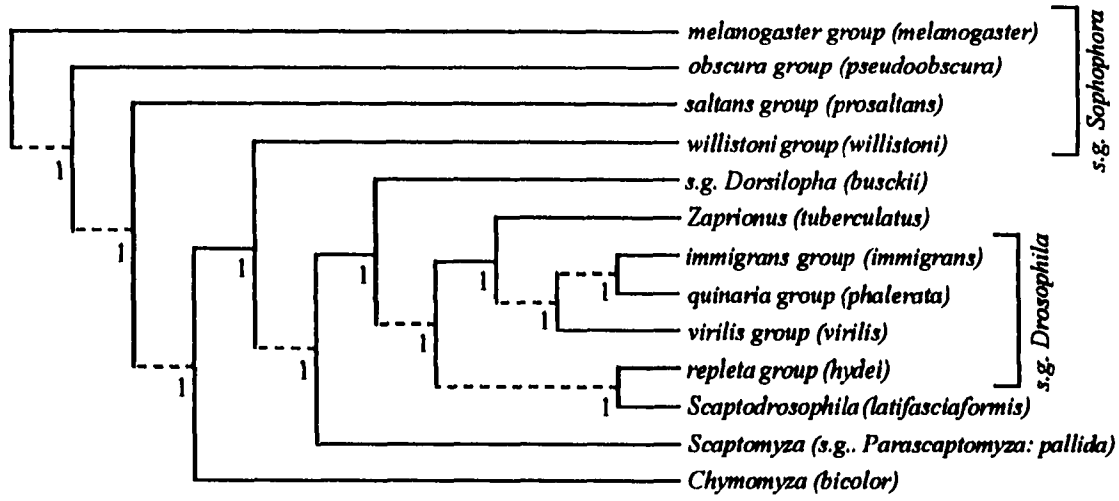
C. *nuDNA Partitions*
1 MPT; 1369 Steps; 393 PICs
CI = 0.793; RI = 0.344

D. *mtDNA Partitions*
1 MPT; 468 Steps; 126 PICs
CI = 0.823; RI = 0.428



E. *All Partitions*
1 MPT; 1914 Steps; 536 PICs
CI = 0.773; RI = 0.338

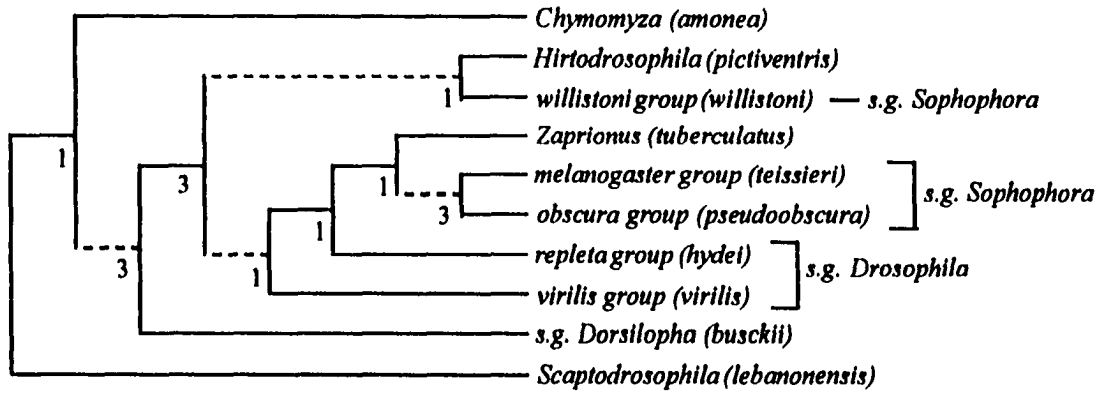
Figure 4



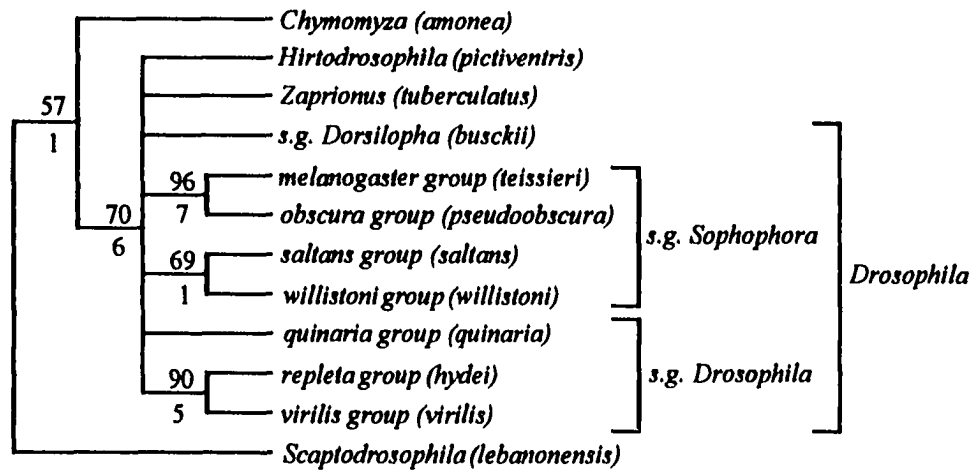
A. *28S - 5+ Partitions*
1 MPT; 232 Steps; 48 PICs
CI = 0.647; RI = 0.410

B. *Adh - 5+ Partitions*
1 MPT; 813 Steps; 246 PICs
CI = 0.637; RI = 0.425

Figure 4

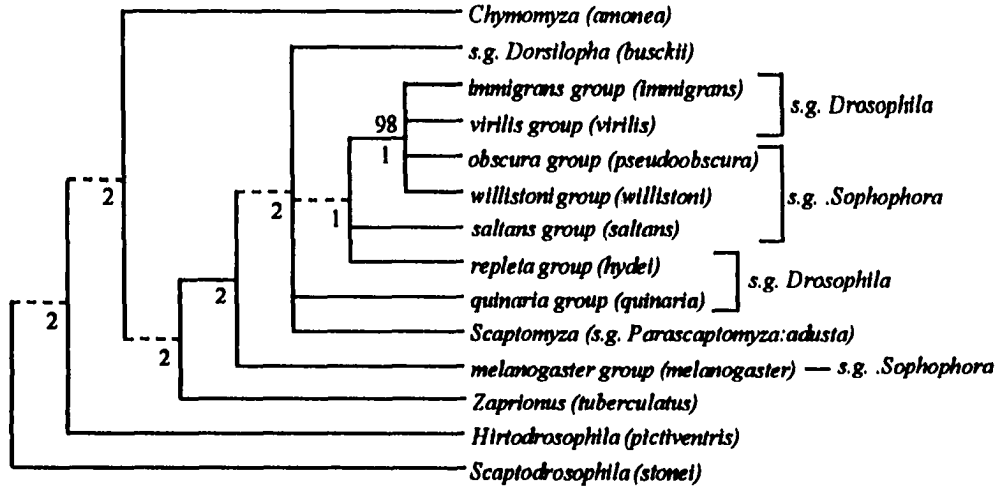


C. *Gpdh - 5+ Partitions*
1 MPT; 820 Steps; 215 PICs
CI = 0.550; RI = 0.279

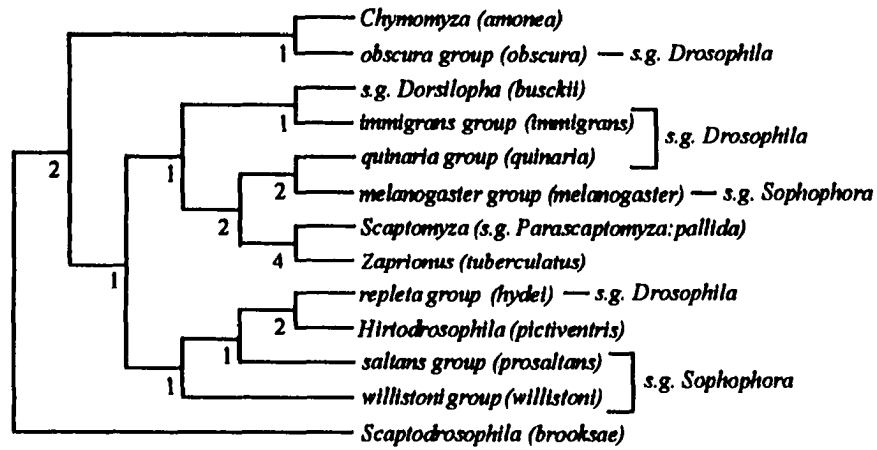


D. *Sod - 5+ Partitions*
4 MPTs; 705 Steps; 176 PICs
CI = 0.502; RI = 0.350

Figure 4



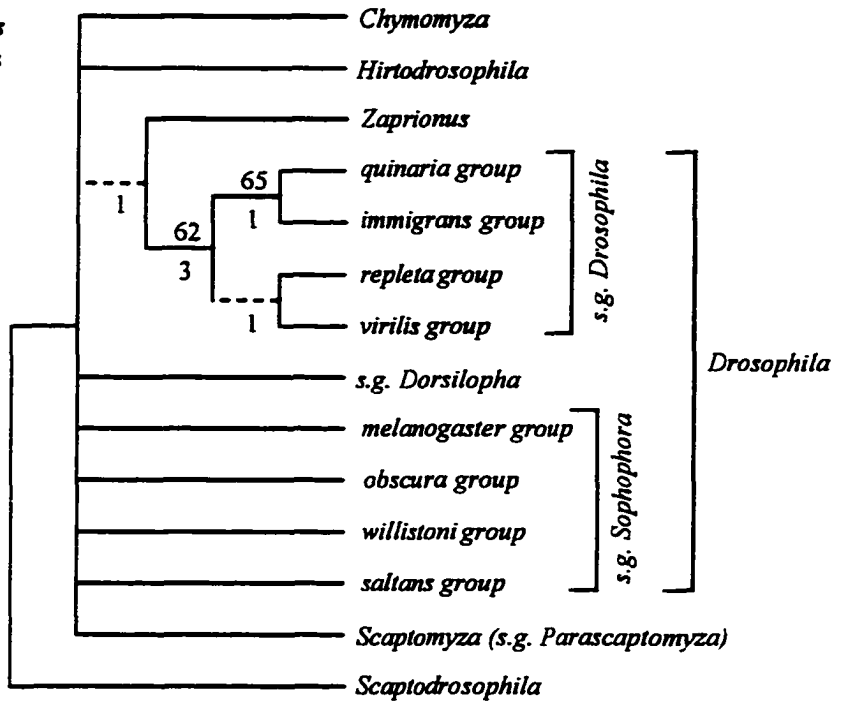
E. *16S - 5+ Partitions*
31 MPTs; 343 Steps; 147 PICs
CI = 0.703; RI = 0.641



F. *COII - 5+ Partitions*
1 MPT; 670 Steps; 162 PICs
CI = 0.497; RI = 0.278

Figure 5

A. *Morphology - 5+ Partitions*
62 MPTs; 140 Steps; 39 PICs
2 MPIs (71% and 29%)
CI = 0.685; RI = 0.542



B. *Molecular - 5+ Partitions*
1 MPT; 3757 Steps; 994 PICs
1 MPI (46%)
6 nMPIs (54% - 3753-3776)
CI = 0.546; RI = 0.306

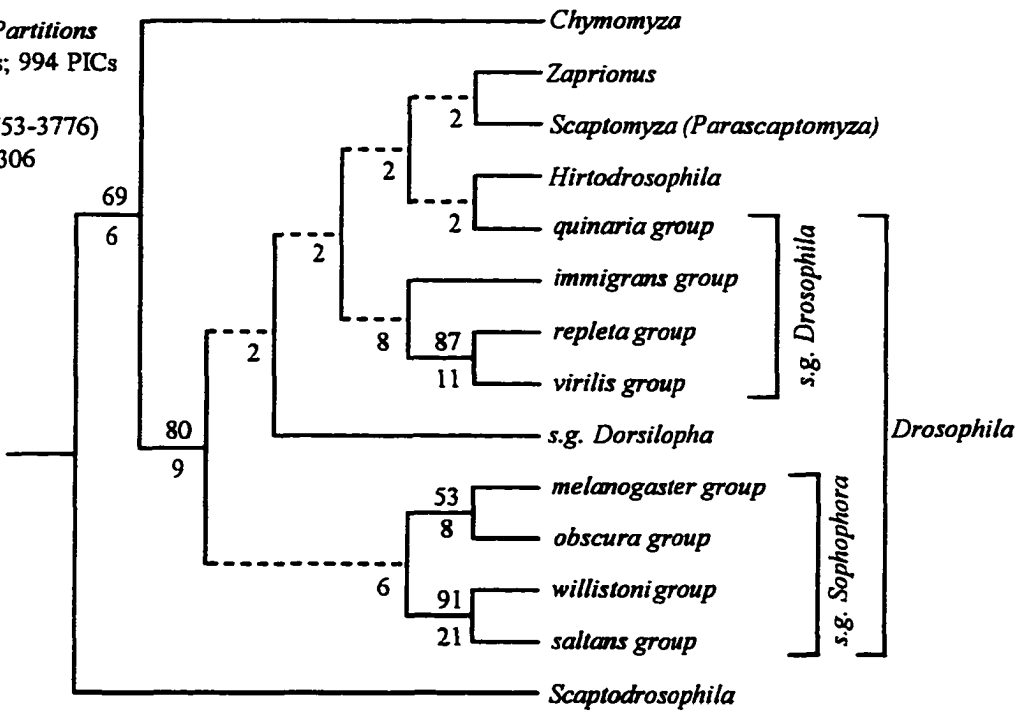
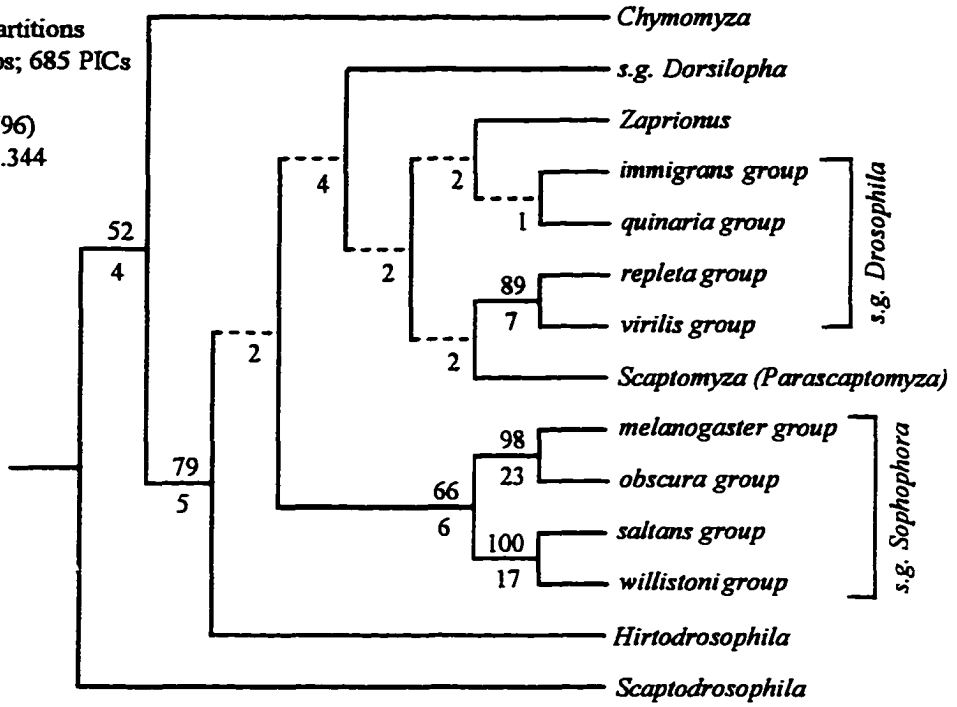


Figure 5

C. nuDNA - 5+ Partitions
1 MPT; 2590 Steps; 685 PICs
1 MPI (84%)
1 nMPI (16% - 2596)
CI = 0.569; RI = 0.344



D. mtDNA - 5+ Partitions
9 MPTs; 1041 Steps; 309 PICs
2 MPIs (47%)
15 nMPIs (53% - 1043-1050)
CI = 0.552; RI = 0.378

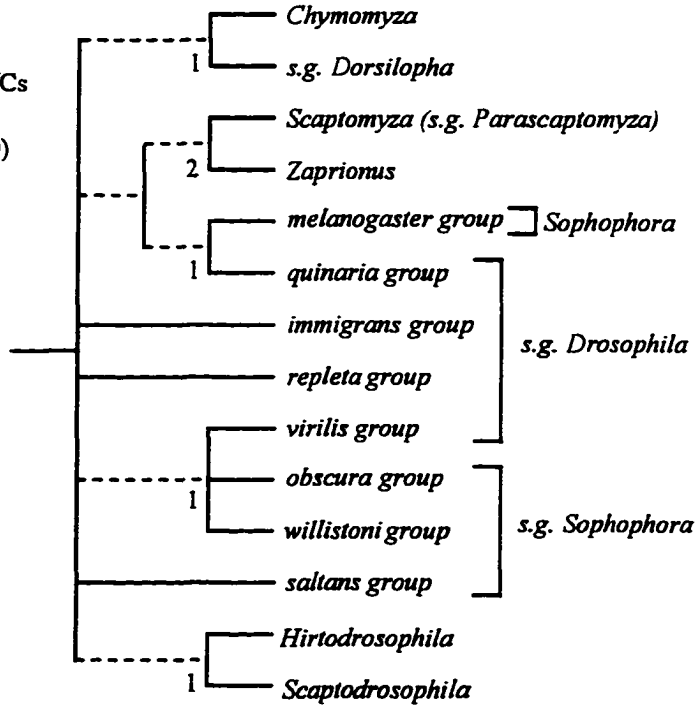


Figure 5

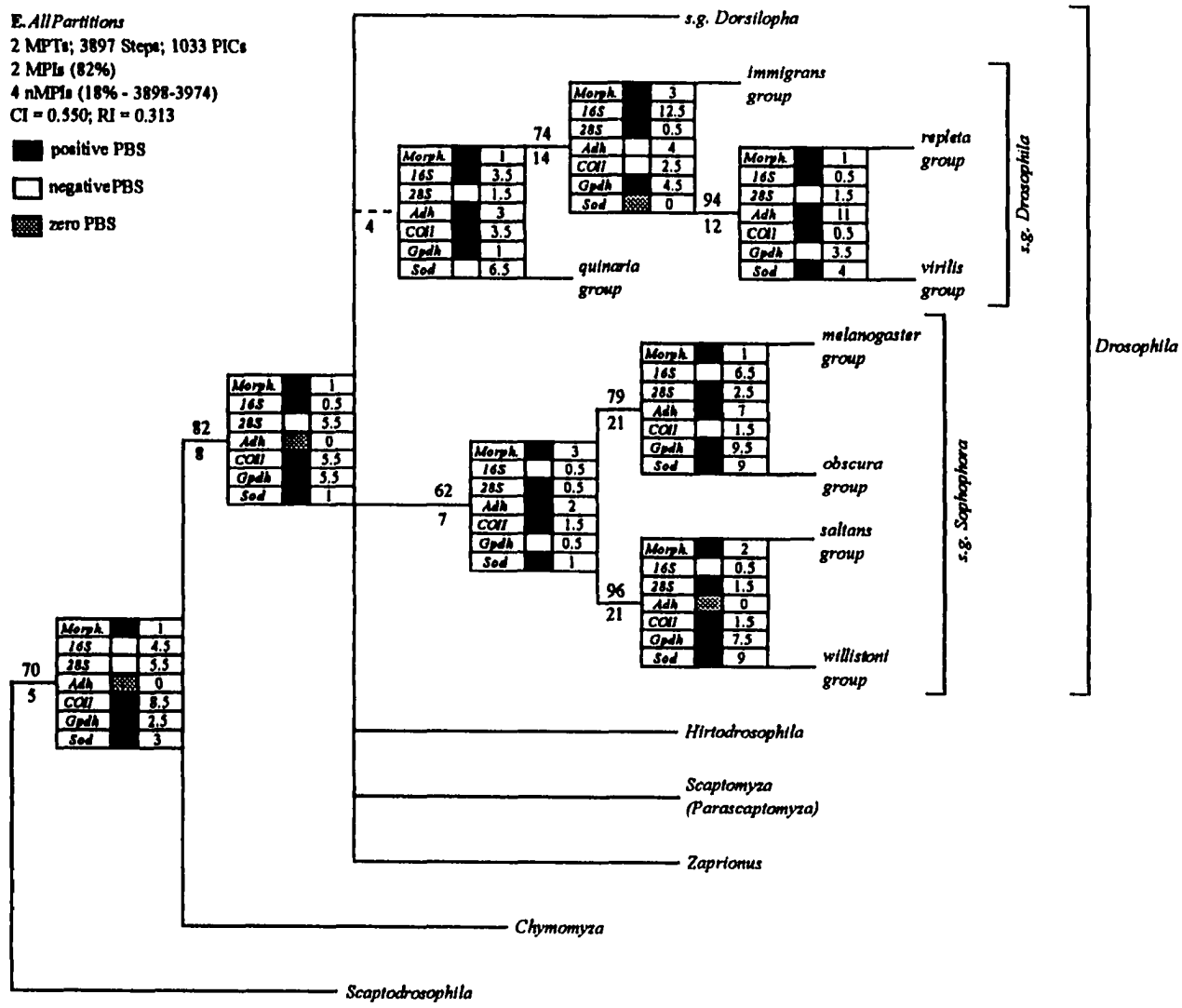
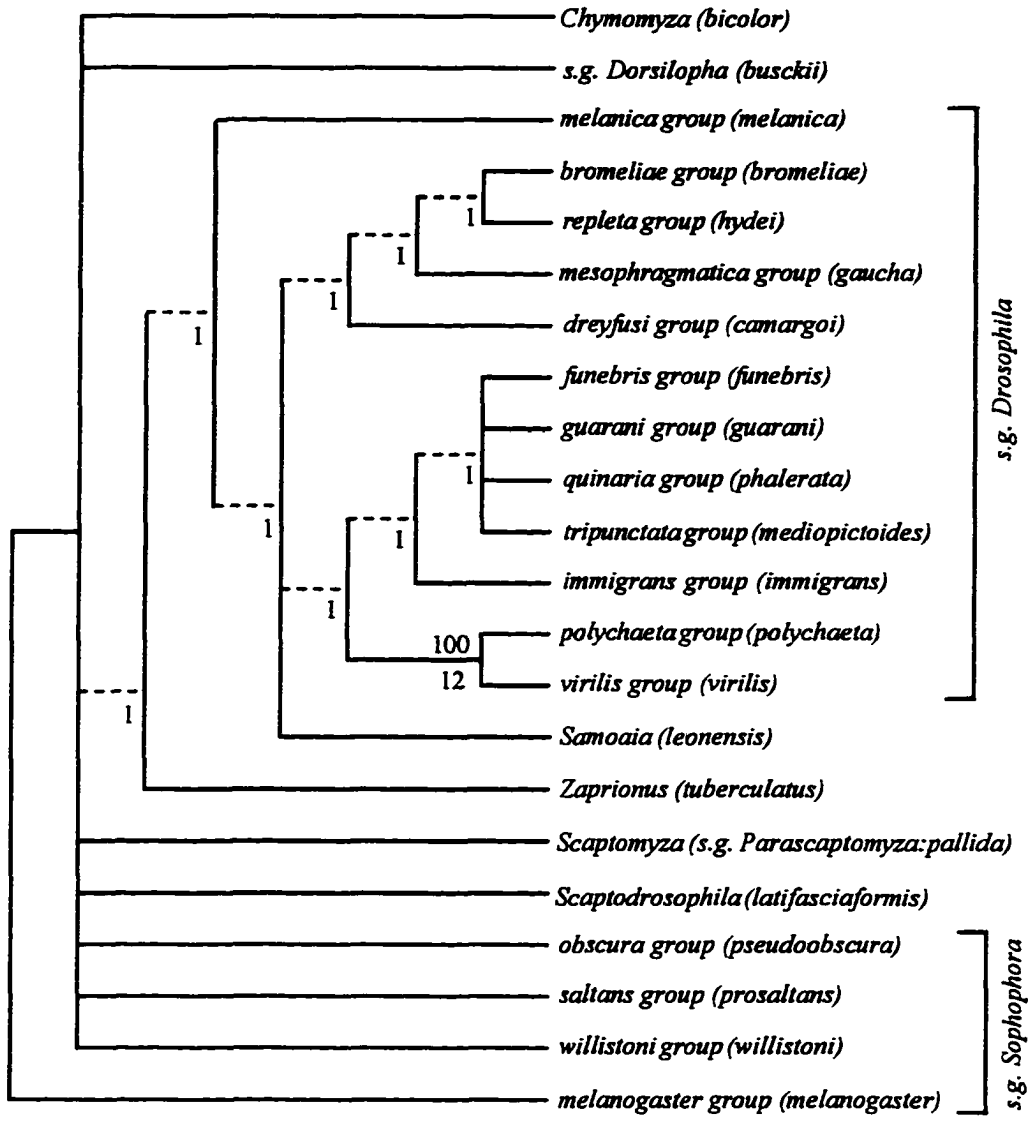
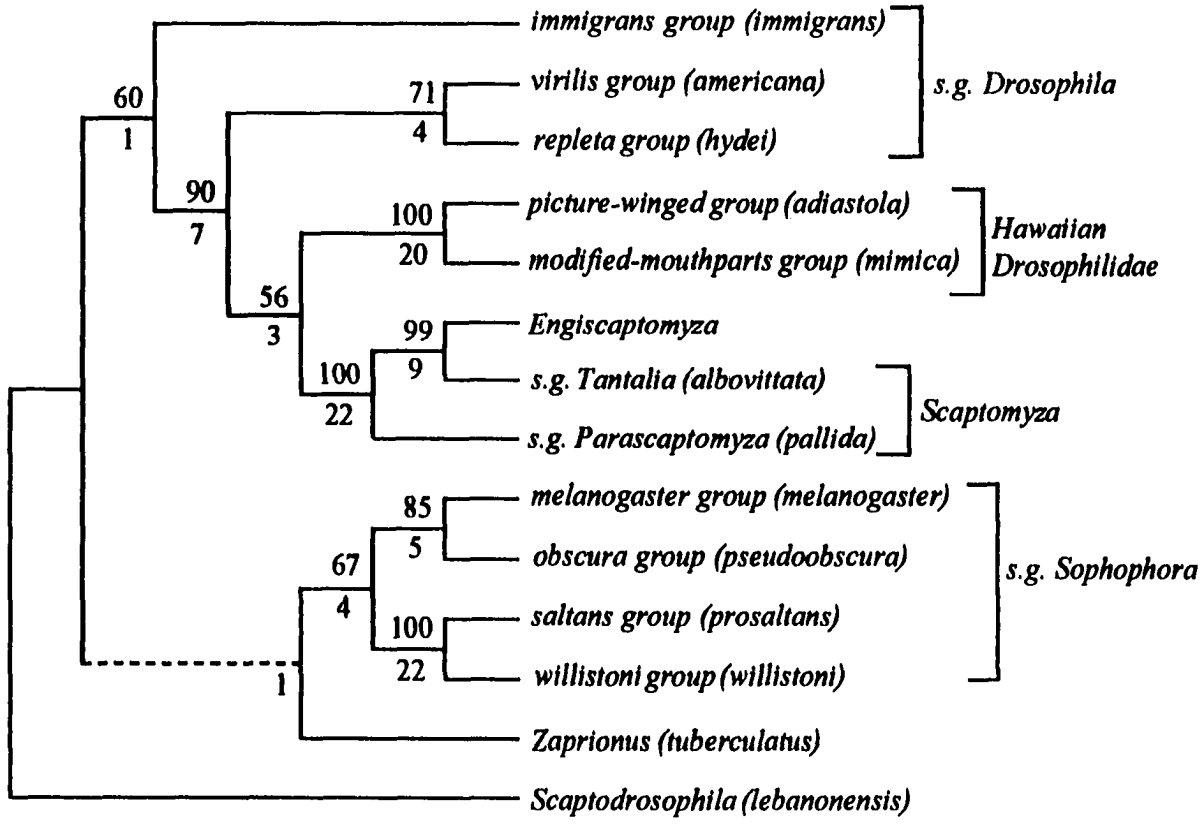

E. All Partitions
2 MPTs; 3897 Steps; 1033 PICs
2 MPIs (82%)
4 nMPIs (18% - 3898-3974)
CI = 0.550; RI = 0.313
positive PBS
negative PBS
zero PBS
s.g. Dorsilopha
immigrans group
repleta group
quinaria group
virilis group
s.g. Drosophila
melanogaster group
obscura group
saltans group
willistoni group
s.g. Sophophora
Drosophila
Hirtodrosophila
Scaptomyza (Parascaptomyza)
Zaprionus
Chymomyza
Scaptodrosophila

Figure 6



A. *28S - 3+ Partitions*
24 MPTs; 343 Steps; 74 PICs
1 MPI (76%)
9 nMPIs (26% - 344-358)
CI = 0.560; RI = 0.493

Figure 6



B. ***Adh- 3+ Partitions***
1 MPT; 1021 Steps; 301 PICs
1 MPI (100%)
CI = 0.574; RI = 0.502

Figure 6

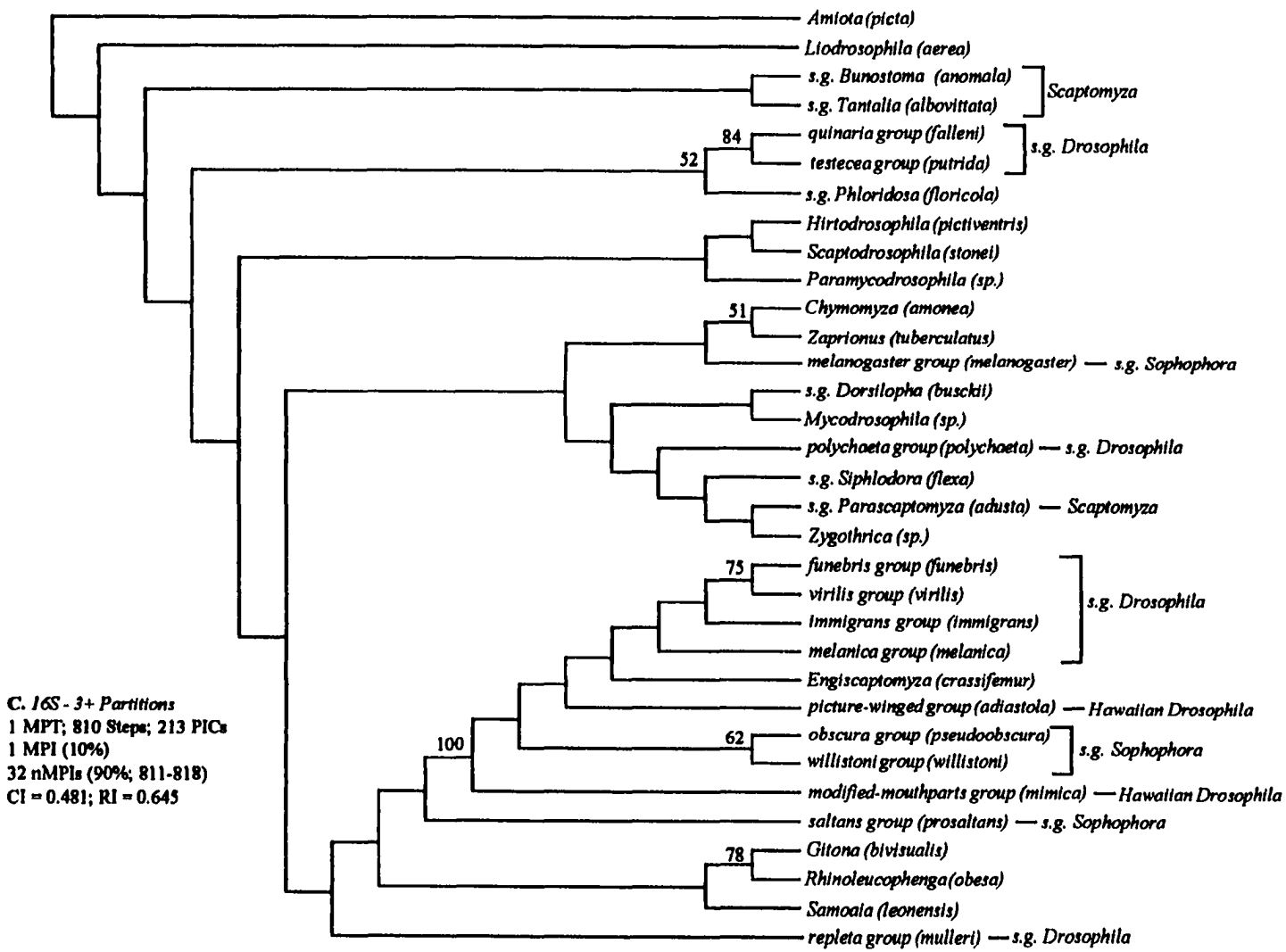
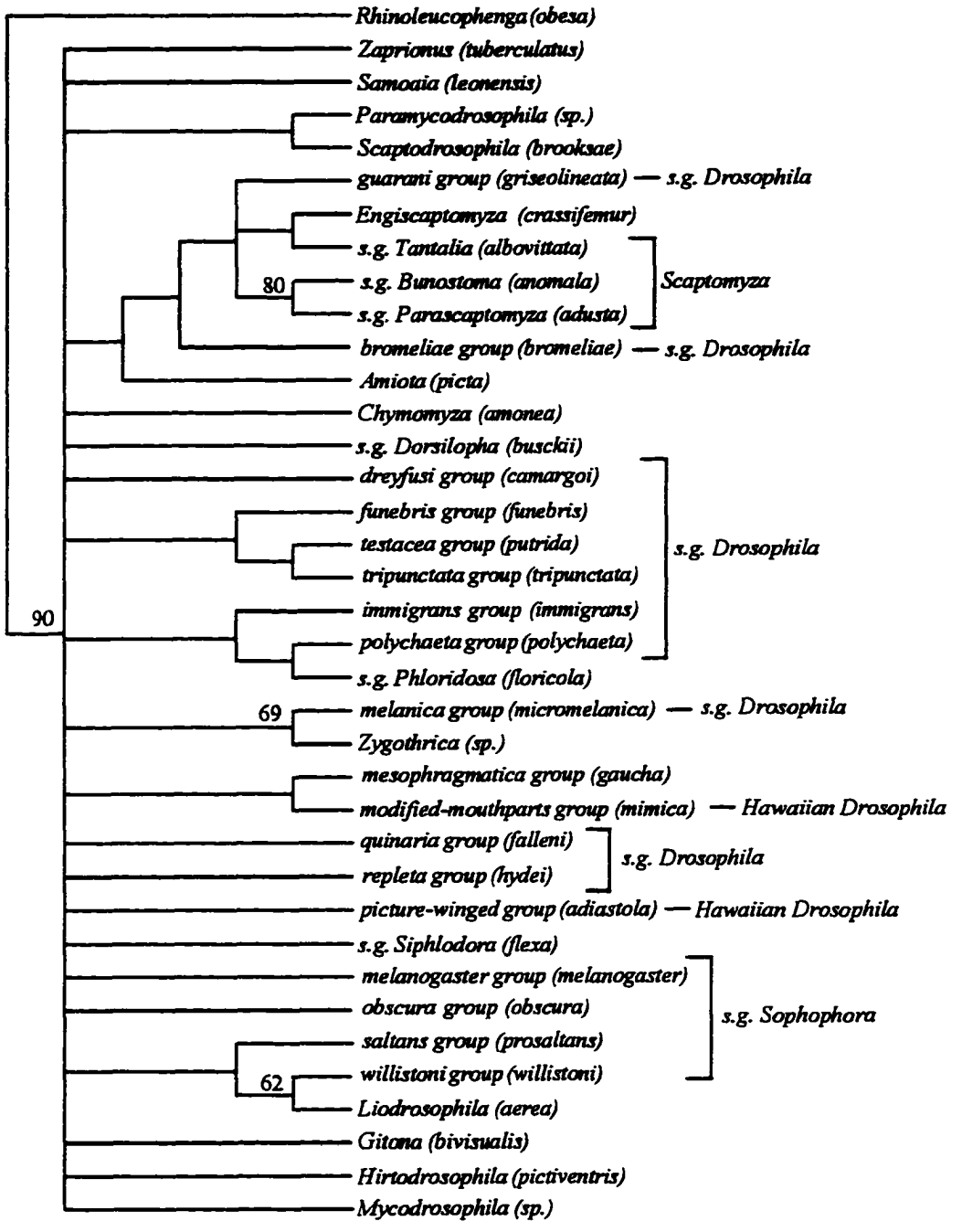
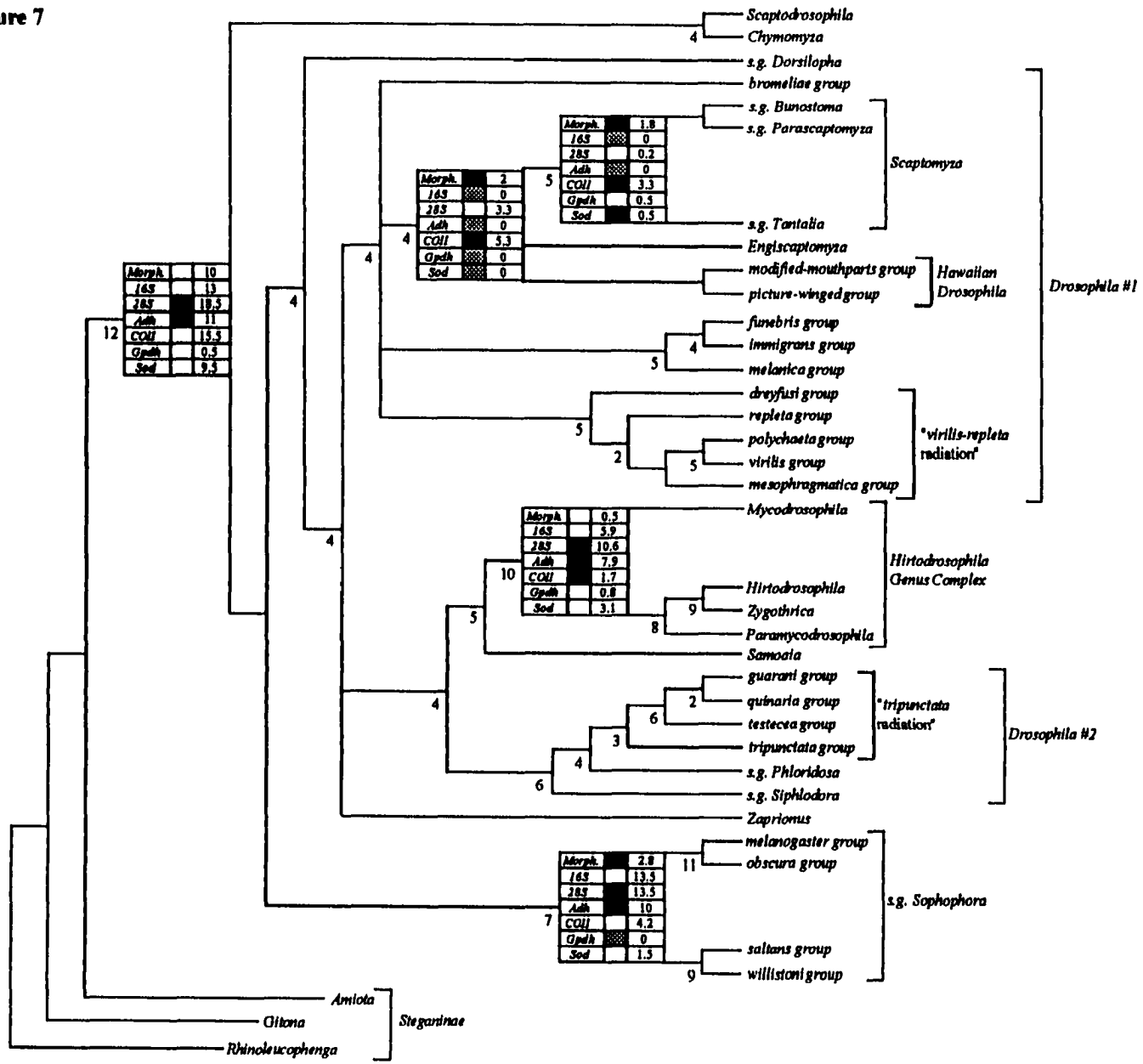

Amiota (picta)
Liodrosophila (aerea)
s.g. Bunostoma (anomala)
s.g. Tantalia (albovittata)
Scaptomyza
84
quinaria group (falleni)
52
testecea group (putrida)
s.g. Drosophila
s.g. Phloridosa (floricola)
Hirtodrosophila (pictiventris)
Scaptodrosophila (stonei)
Paramycodrosophila (sp.)
51
Chymomyza (amonea)
Zaprionus (tuberculatus)
melanogaster group (melanogaster) — s.g. Sophophora
s.g. Dorsilopha (buscki)
Mycodrosophila (sp.)
polychaeta group (polychaeta) — s.g. Drosophila
s.g. Siphlodora (flexa)
s.g. Parascaptomyza (adusta) — Scaptomyza
Zygothrica (sp.)
75
funebris group (funebris)
virilis group (virilis)
immigrans group (immigrans)
melanica group (melanica)
s.g. Drosophila
Engiscaptomyza (crassifemur)
picture-winged group (adiastola) — Hawaiian Drosophila
62
obscura group (pseudoobscura)
willistoni group (willistoni)
s.g. Sophophora
100
modified-mouthparts group (mimica) — Hawaiian Drosophila
saltans group (prosaltans) — s.g. Sophophora
78
Gitona (bivisualis)
Rhinoleucophenga (obesa)
Samoaia (leonensis)
repleta group (mulleri) — s.g. Drosophila
C. 16S - 3+ Partitions
1 MPT; 810 Steps; 213 PICs
1 MPI (10%)
32 nMPIs (90%; 811-818)
CI = 0.481; RI = 0.645

Figure 6



D. *COII - 3+ Partitions*
18 MPTs; 1669 Steps; 237 PICs
6 MPIs (6%)
88 nMPIs (94% - 1670-1688)
CI = 0.327; RI = 0.313

Figure 7


Scaptodrosophila
Chymomyza
s.g. Dorsilopha
bromeliae group
s.g. Bunostoma
s.g. Parascaptomyza
Scaptomyza
s.g. Tantalia
Engiscaptomyza
modified-mouthparts group
picture-winged group
Hawaiian Drosophila
funebris group
immigrans group
melanica group
dreyfusi group
repleta group
polychaeta group
virilis group
mesophragmatica group
"virilis-repleta radiation"
Drosophila #1
Mycodrosophila
Hirtodrosophila
Zygothrica
Paramycodrosophila
Hirtodrosophila Genus Complex
Samoaia
guarani group
quinaria group
testacea group
tripunctata group
s.g. Phloridosa
s.g. Siphlodora
"tripunctata radiation"
Drosophila #2
Zaprionus
melanogaster group
obscura group
saltans group
willistoni group
s.g. Sophophora
Amiota
Gitona
Rhinoleucophenga
Steganinae

Appendix 1

A list of species used in the present study is shown below. Genera, following the taxonomic classification of Wheeler (1981; 1986), are listed alphabetically. Subgenera and species groups are also listed, when appropriate. Morphological studies list the first author (in bold), followed by the species examined (when available) and the number of characters examined. Molecular studies list the first author (in bold) followed by the locus examined and the genbank accession number. Sequences marked with an (*) were generated in this study. Collection information or the source of the flies examined in the present studyis also included. Numbers in parentheses preceeded by an O refer to the author's collection notes, which are available on request.

Ingroup Species

Amiota

Okada: species not stated - *"Amiota"* (14 characters); **Grimaldi**: *picta* (213 characters); **O'Grady**: *picta* (*16S*: XXX*; *COII*: XXX*).

Specimen Information: **O'Grady**: Oversite Canyon, Huachuca Mountains, AZ; 15 May 1995. Nine individuals were aspirated from about the head of the collector in a pine/oak forest.

Chymomyza

Throckmorton: species not stated - *"Chymomyza"* (18 characters); **Okada**: species not stated - *"Chymomyza"* (14 characters); **Grimaldi**: *amonea* (218 characters); **DeSalle**: *amonea* (*16S*: M93986); **Pelendakis**: *bicolor* (*28S*: X71299); **Kwiatowski:** *amonea* (*Gpdh*: L36961; *Sod*: X61687); **O'Grady**: *amonea* (*COII*: XXX*).

Specimen Information: Representatives of *C. amonea* were obtained from the National *Drosophilia* Species Stock Center (20000-2621.0).

Drosophila (*Dorsilopha*)

Throckmorton: *busckii* (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *busckii* (218 characters); **Pelendakis**: *busckii* (*28S*: X71273); **Kwiatowski**: *busckii* (*Gpdh*: AF039630; *Sod*: U39445); **O'Grady**: *busckii* (*16S*: XXX*; *COII*: XXX*).

Specimen Information: **O'Grady**: San Quintin, Baja California Norte, Mexico; November 1995. Twenty four individuals were aprirated from rotting fruit in a trash can.

Drosophila (*Drosophila*) - *bizonata* species group

Okada: species not stated - "*Drosophila*" (14 characters); **O'Grady** (*16S*: XXX*; *COII*: XXX*).

Specimen Information: **Watada**:

Drosophila (*Drosophila*) - *bromeliae* species group

Throckmorton: species not stated - "*D. bromeliae* group" (17 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Pelendakis**: *bromeliae* (*28S*: X71235); **O'Grady**: *bromeliae* (*COII*: XXX*).

Specimen Information: Representatives of *D. bromeliae* were obtained from the National *Drosophilia* Species Stock Center (15088-1682.0).

Drosophila (*Drosophila*) - *calloptera* species group

Throckmorton: species not stated - "*D. calloptera* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: calloptera (218 characters); **O'Grady**: *ornatipennis* (*COII*: XXX*).

Specimen Information: Representatives of *D. ornatipennis* were obtained from the National *Drosophilia* Species Stock Center (15160-2121.0).

Drosophila (*Drosophila*) - *cardini* species group

Throckmorton: species not stated - "*D. cardini* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **O'Grady**: *dunni* (*COII*: XXX*).

Specimen Information: Representatives of *D. dunni* were obtained from the National *Drosophilia* Species Stock Center (15182-2291.0).

Drosophila (*Drosophila*) - *dreyfusi* species group

Throckmorton: species not stated - "*D. dreyfusi* group" (17 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Pelendakis**: *carmagoi* (*28S*: X71245); **O'Grady**: *camargoi* (*COII*: XXX*).

Specimen Information: Representatives of *D. camargoi* were obtained from the National *Drosophilia* Species Stock Center (15060-1221.0). **O'Grady**: Jatun Sacha Biological Station, Tena, Ecuador; 5 August 1997 (O28.10). Approximately 40 individuals were collected by sweeping over rotting fruit. A stock has been established and is in culture in the Heed Laboratory.

Drosophila (*Drosophila*) - *funebris* species group

Throckmorton: species not stated - "*D. funebris* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *funebris* (217 characters); **DeSalle**: *funebris* (*16S*: not listed in genbank); **Pelendakis**: *funebris* (*28S*: X71227); **O'Grady**: *funebris* (*COII*: XXX*).

Specimen Information: Representatives of *D. funebris* were obtained from the National *Drosophilia* Species Stock Center (15120-1911.6).

Drosophila (*Drosophila*) *guarani* species group

Throckmorton: species not stated - "*D. guarani* group" (17 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Pelendakis**: *guarani* (*28S*: X71259); **O'Grady**: *griseolineata* (*COII*: XXX*).

Specimen Information: Representatives of *D. griseolineata* were obtained from the National *Drosophilia* Species Stock Center (15171-2131.3).

Drosophila (*Drosophila*) *histrio* species group

Okada: species not stated - *"Drosophila"* (14 characters); **Pelendakis**: *sternopleuralis* (*28S*: X71263); **O'Grady**: *sternopleuralis* (*COII*: XXX*).

Specimen Information: Representatives of *D. sternopleuralis* were obtained from the National *Drosophilia* Species Stock Center (15270-2461.0).

Drosophila (*Drosophila*) *immigrans* species group

Throckmorton: *immigrans* (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *immigrans* (218 characters); **DeSalle**: *immigrans* (*16S*: M93988); **Abalot** and **Gonzalez-Duatre**: *immigrans* (*Adh*: M97638); **Pelendakis**: *immigrans* (*28S*: X71231); **O'Grady**:*immigrans* (*COII*:XXX*).

Specimen Information: **O'Grady**: Tucson, AZ; 12 February 1995. Approximately 20 individuals were collected by sweeping over a compost heap at 2842 East Stratford Drive. A stock was established for DNA isolation and was subsequently lost.

Drosophila (*Drosophila*) *macroptera* species group

Throckmorton: species not stated - "*D. macroptera* group" (16 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **O'Grady**: *macroptera* (16S: XXX*).

Specimen Information: **Heed, Babcock, Dyreson, Silva** and **O'Grady**: Mt. Lemmon, AZ; 25 August 1997 (O29.3). Eleven individuals were either aspirated from a variety of fungus or collected with fermenting banana bait in the vicinity of Dr. Heed's cabin.

Drosophila (*Drosophila*) *melanica* species group

Throckmorton: species not stated - "*D. melanica* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *melanica* (206 characters); **DeSalle**: *melanica* (*16S*: M93989); **Pelendakis**:*melanica* (*28S*: X71233); **O'Grady**: *micromelanica* (*COII* XXX*).

Specimen Information: Representatives of *D. micromelanica* were obtained from the National *Drosophilia* Species Stock Center (15030-1151.0).

Drosophila (*Drosophila*) *mesophragmatica* species group

Throckmorton: species not stated - "*D. mesophragmatica* group" (16 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Pelendakis**:*gaucha* (*28S*: X71253); **O'Grady**: *gaucha* (*COII* XXX*).

Specimen Information: Representatives of *D. gaucha* were obtained from the National *Drosophilia* Species Stock Center (15070-1231.0).

Drosophila (*Drosophila*) *nannoptera* species group

Throckmorton: species not stated - "*D. nannoptera* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **O'Grady**: *nannoptera* (*COII*: XXX*).

Specimen Information: Representatives of *D. nannoptera* were obtained from the Heed Laboratory.

Drosophila (*Drosophila*) *polychaeta* species group

Throckmorton: *D. polychaeta* (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Pelendakis**:*polychaeta* (*28S*: X71225); **O'Grady**: *polychaeta* (16S: XXX*; *COII* XXX*).

Specimen Information: Representatives of *D. polychaeta* were obtained from the National *Drosophilia* Species Stock Center (15100-1711.0).

Drosophila (*Drosophila*) *quinaria* species group

Throckmorton: species not stated - "*D. quinaria* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Pelendakis**: *phalerata* (*28S*: X71255); **Kwiatowski**: *quinaria* (*Sod*: not listed in genbank); **O'Grady**: *falleni* (16S: XXX*), *quinaria* (*COII* XXX*).

Specimen Information: Representatives of *D. falleni* were obtained from the National *Drosophilia* Species Stock Center (15100-1711.0). **O'Grady**: *quinaria*; Whitesboro, NY; 1 August 1996. Three individuals of *D. quinaria* were swept over rotting vegetation. **O'Grady**: *falleni*; Trenton Falls Gorge, Trenton Falls, NY; July 1997. Twenty three individuals of *D. falleni* were aspirated from fungus.

Drosophila (*Drosophila*) *repleta* species group

Throckmorton: species not stated - "*D. hydei* subgroup" (17 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *repleta* (218 characters); **DeSalle**: *mulleri* (*16S*: U94247); **Pelendakis**: *hydei* (*28S*: X71249); **Menotti-Raymond**: *hydei* (*Adh-1*: X58694); **Kwiatowski**: *hydei* (*Gpdh*: L41650; *Sod*: Z14144); **O'Grady**: *hydei* (*COII* XXX*).

Specimen Information: Representatives of *D. hydei* were obtained from the Heed Laboratory.

Drosophila (*Drosophila*) *robusta* species group

Throckmorton: species not stated - "*D. robusta* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *colorata* (218 characters); **Pelendakis**: *robusta* (*28S*: X71237); **DeSalle**: *robusta* (*16S*: M93994).

Drosophila (*Drosophila*) *rubifrons* species group

Throckmorton: species not stated - "*D. rubifrons* group" (16 characters); **Okada**: species not stated - *"Drosophila"* (14 characters).

Specimen Information: **O'Grady**: Mt. Bigelow, AZ; September 1996. Three individuals were aspiarated from mushrooms. **Heed, Babcock, Dyreson, Silva** and **O'Grady**: Mt. Lemmon, AZ; 25 August 1997 (O29.2). Forty three individuals were either aspirated from a variety of fungus or collected with fermenting banana bait in the vicinity of Dr. Heed's cabin.

Drosophila (*Drosophila*) *testacea* species group

Throckmorton: species not stated - "*D. testacea* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *testacea* (218 characters); **O'Grady**: *putrida* (*16S*: XXX* *COII*: XXX*).

Specimen Information: Representatives of *D. putrida* were obtained from the National *Drosophilia* Species Stock Center (15150-2101.0).

Drosophila (*Drosophila*) *tripunctata* species group

Throckmorton: species not stated - "*D. tripunctata* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *tripunctata* (218 characters); **Pelendakis**: *mediopictoides* (*28S*: X71265); **O'Grady**: *tripunctata* (*COII*: XXX*).

Specimen Information: **O'Grady** and **Dyreson**: Athens, Ga; June 1994. Approximately 10 flies were aspirated from fungus. A stock was started for DNA and it has been lost.

Drosophila (*Drosophila*) *tumiditarsus* species group

Okada: species not stated - *"Drosophila"* (14 characters); **O'Grady**: *repletoides* (*COII*: XXX*).

Specimen Information: Representatives of *D. repletoides* were obtained from the National *Drosophilia* Species Stock Center (15250-2451.0).

Drosophila (*Drosophila*) *virilis* species group

Throckmorton: species not stated - "*D. virilis* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *virilis* (217 characters); **DeSalle**: *virilis* (*16S*: not listed in genbank); **Pelendakis**: *mediopictoides* (*28S*: X71239);

Kwiatowski: *virilis* (*Gpdh*: X59076; *Sod*: X13831) **Numirsky**: *americana* (Adh: U26844).

Drosophila (Hawaiian *Drosophila*) *antopocerus* species group

Throckmorton: species not stated - "*Antopocerus*" (18 characters); **Okada**: species not stated - "*Drosophila*" (14 characters); **Grimaldi**: *adunca* (217 characters); **DeSalle**: *adunca* (*16S*: U94241; *COII*: U94240).

Drosophila (Hawaiian *Drosophila*) *fungus-feeders* species group

Throckmorton: species not stated - "Hawaiian *Drosophila*" (18 characters); **Okada**: species not stated - "*Drosophila*" (14 characters); **Thomas** and **Hunt**: *nigra* (Adh: M60793).

Drosophila (Hawaiian *Drosophila*) *modified-mouthparts* species group

Throckmorton: species not stated - "Hawaiian *Drosophila*" (18 characters); **Okada**: species not stated - "*Drosophila*" (14 characters); **Grimaldi**: *mimica* (217 characters); **DeSalle**: *mimica* (*16S*: not listed in genbank; *COII*: U94217). **Thomas** and **Hunt**: *mimica* (*Adh*: M60792).

Drosophila (Hawaiian *Drosophila*) *modified-tarsus* species group

Throckmorton: species not stated - "Hawaiian *Drosophila"* (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **DeSalle**: *dasycnemia* (*16S*: U94253; *Adh*: U94208; *COII*: U94224).

Drosophila (Hawaiian *Drosophila*) *picture-winged* species group

Throckmorton: species not stated - "Hawaiian *Drosophila"* (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *adiastola* (204 characters); **DeSalle**: *adiastola* (*16S*: S45476; *COII*: U94209); **Thomas** and **Hunt**: *adiastola* (*Adh*: M60791).

Drosophila (Hawaiian *Drosophila*) *white-tipped scutellum* species group

Throckmorton: species not stated - "Hawaiian *Drosophila"* (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **DeSalle**: *iki* (*16S*: U94244; *Adh*: U94198; *COII*: U94214).

Drosophila (*Lordiphosa*)

Okada: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *fenestratum* (217 characters); **Pelendakis**: *andalousiaca* (*28S*: XXX).

Drosophila (*Phloridosa*)

Throckmorton: species not stated - *"Phloridosa"* (16 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *floricola* (218 characters); **O'Grady**: *floricola* (*16S*: XXX*; *COII*: XXX*).

Specimen Information: **O'Grady** and **Salywon**: La Aduana, Sonora, Mexico; 24 December 1994. Thriry six flies were aspirated from fallen flowers of a morning glory tree. **O'Grady**: Malibu, Ca.; October 1995. One individual was aspirated from flowers. **O'Grady**: Santo Tomas, Baja California Norte, Mexico; November 1995. Fifty nine individuals were aspirated from Datura flowers growing in the wash of the Rio Santo Tomas.

Drosophila (*Siphlodora*)

Okada: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *flexa* (218 characters); **O'Grady**: *flexa* (*16S*: XXX*; *COII*: XXX*).

Specimen Information: **Babcock, Silva,** and **O'Grady**: El Caracol, Sonora, Mexico; 1-2 May 1997. Twenty five individuals were collected with fermenting banana bait.

Drosophila (*Sophophora*) *melanogaster* species group

Throckmorton: species not stated - *"S. melanogaster* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *melanogaster* (218

characters); **Kreitman**: *melanogaster* (*Adh*: M17833); **DeSalle**: *melanogaster* (*16S*: XXX); **Pelendakis**: *melanogaster* (*28S*: XXX); **deBruijn**: *melanogaster* (*COII*: XXX); **Kwiatowski**: *teissieri* (*Gpdh*: XXX; *Sod*: XXX).

Drosophila (*Sophohpora*) *obscura* species group

Throckmorton: species not stated - "*S. obscura* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Grimaldi**: *affinis* (218 characters); **DeSalle**: *pseudoobscura* (*16S*: XXX); **Marfany** and **Gonzalez-Duarte**: *ambigua* (*Adh*: X54813); **Pelendakis**: *pseudoobscura* (*28S*: XXX); **Kwiatowski**: *pseudoobscura* (*Gpdh*: XXX; *Sod*: XXX); **O'Grady**: *obscura* (*COII*: XXX*).

Specimen Information: Specimens of *D. obscura* were obtained from Dr. M. Radak at the University of Belgrade.

Drosophila (*Sophophora*) *saltans* species group

Throckmorton: species not stated - "*S. saltans* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **Pelendakis**: *prosaltans* (*28S*: XXX); **Kwiatowski**: *saltans* (*Sod*: XXX); **O'Grady**: *prosaltans* (*16S*: XXX*; *Adh*: AF045118*; *COII*: AF045087*).

Specimen Information: Representatives of *D. prosaltans* were obtained from the National *Drosophilia* Species Stock Center (14045-0901.0).

Drosophila (*Sophophora*) *willistoni* species group

Throckmorton: species not stated - "*S. willistoni* group" (18 characters); **Okada**: species not stated - *"Drosophila"* (14 characters); **DeSalle**: *willistoni* (*16S*: XXX); **Anderson**: *willistoni* (*Adh*: L08648); **Pelendakis**: *willistoni* (*28S*: XXX); **Kwiatowski**: *willistoni* (*Gpdh*: XXX; *Sod*: XXX); **O'Grady**: *willistoni* (*COII*: XXX*).

Specimen Information: Representatives of *D. willistoni* were obtained from the National *Drosophilia* Species Stock Center (14030-0811.0).

Engiscaptomyza

Throckmorton: *crassifemur* (18 characters); **Grimaldi**: *crassifemur* (218 charactrers); **DeSalle**: *crassifemur* (*16S*: XXX; COII: XXX); **Thomas** and **Hunt**: *crassifemur* (*Adh*: M60790).

Gitona

Okada: species not stated - *"Gitona"* (14 characters); **Grimaldi**: *bivisualis* (218 characters); **O'Grady**: *bivisualis* (*16S*: XXX*; *COII*: XXX*).

Specimen Information: **O'Grady**: Empire-Cienega Riparian Reserve, Sonoita, AZ; 10-11 November 1996 (1.3). Twenty nine individuals were collected using fermenting banana bait.

Hirtodrosophila

Throckmorton: species not stated - "*Hirtodrosophila*" (18 characters); **Okada**: species not stated - "*Hirtodrosophila*" (14 characters); **Grimaldi**: *nigrohaltera* (218 characters); **DeSalle**: *pictiventris* (*16S*: XXX); **Kwiatowski**: *pictiventris* (*Gpdh*: XXX; *Sod*: XXX); **O'Grady**: *pictiventris* (*COII*: XXX*).

Specimen Information: Representatives of *H. pictiventris* were obtained from the National *Drosophilia* Species Stock Center (12000-0072.0).

Leucophenga

Okada: species not stated - "*Leucophenga*" (14 characters); **Grimaldi**: *maculata* (218 characters); **Pelendakis**: *maculata* (28S: XXX).

Liodrosophila

Throckmorton: species not stated - "*Liodrosophila*" (18 characters); **Okada**: species not stated - "*Liodrosophila*" (14 characters); **Grimaldi**: *onchopyga* (218 characters); **DeSalle**: *aerea* (*16S*: XXX); **O'Grady**: *aerea* (*COII*: XXX*).

Specimen Information: Representatives of *L. aerea* were obtained from the National *Drosophilia* Species Stock Center (60000-2751.0).

Mycodrosophila

Throckmorton: species not stated - *"Mycodrosophila"* (18 characters); **Okada**: species not stated - *"Mycodrosophlia"* (14 characters); **Grimaldi**: *dimitada* (218 characters); **O'Grady**: *sp. 157* (*16S*: XXX*; *COII*: XXX*).

Specimen Information: **O'Grady**: near Mapledale Road, Barneveld, NY; July 1997 (24.1). Eight specimens were aspirated from bracket fungus in a pine forest.

Paramycodrosophila

Okada: species not stated - *"Paramycodrosophila"* (14 characters); **Grimaldi**: *sp* (218 characters); **O'Grady**: *sp. 181* (*16S*: XXX*; *COII*: XXX*).

Specimen Information: **O'Grady**: Jatun Sacha Biological Station, Tena, Ecuador; 5 August 1997 (28.3). Two individuals were swept from fungus.

Rhinoleucophenga

Okada: species not stated - *"Rhinoleucophenga"* (14 characters); **Grimaldi**: *obesa* (218 characters); **O'Grady**: *obesa* (*16S*: XXX*; *COII*: XXX*).

Specimen Information: **Etges** and **Huckins**: Chamela, Mexico.

Samoaia

Okada: species not stated - *"Mycodrosophlia"* (14 characters); **Grimaldi**: *ocellaris* (218 characters); **Pelendakis**: *leonensis* (*28S*: XXX); **O'Grady**: *leonensis* (*16S*: XXX*; *COII*: XXX*).

Specimen Information: Representatives of *S. leonensis* were obtained from the National *Drosophilia* Species Stock Center (80000-2761.0).

Scaptodrosophila

Throckmorton: species not stated - "*Scaptodrosophila*" (18 characters); **Okada**: species not stated - *"Scaptodrosophila"* (14 characters); **Grimaldi**: *scaptomyzoidea* (218 characters); **DeSalle**: *stonei* (*16S*: XXX); **Marfany** and **Gonzalez-Duarte**: *lebanonensis* (*Adh*: X54814); **Pelendakis**: *latifasciaformis* (*28S*: XXX); **O'Grady**: *brooksae* (*COII*: XXX*).

Specimen Information: Representatives of *S. brooksae* were obtained from the National *Drosophilia* Species Stock Center (11010-0045.0).

Scaptomyza (*Bunostoma*)

Throckmorton: species not stated - "*Bunostoma*" (18 characters); **Okada**: species not stated - *"Scaptomyza"* (14 characters); **Grimaldi**: *australis* (218 characters); **O'Grady**: *anomala* (*16S*: XXX*; *COII*: XXX*).

Specimen Information: Representatives of *S. anomala* were obtained from the National *Drosophilia* Species Stock Center (33000-2661.0).

Scaptomyza (*Parascaptomyza*)

Throckmorton: species not stated - "*Parascaptomyza*" (18 characters); **Okada**: species not stated - "*Scaptomyza*" (14 characters); **Grimaldi**: *pallida* (218 characters); **Pelendakis**: *pallida* (*28S*: XXX); **Tamura**: *pallida* (*Adh*: unpublished); **O'Grady**: *adusta* (*16S*: XXX*); *pallida* (*COII*: XXX*).

Specimen Information: Representatives of *S.adusta* were obtained from the National *Drosophilia* Species Stock Center (31000-2641.0). Representatives of *S. pallida* were obtained from Dr. Jonathan Clark.

Scaptomyza (*Scaptomyza*)

Throckmorton: species not stated - "*Scaptomyza*" (18 characters); **Okada**: species not stated - "*Scaptomyza*" (14 characters); **Grimaldi**: *graminum* (218 characters); **Tamura**: *gramimum* (*Adh*: unpublished).

Scaptomyza (*Tantalia*)

Throckmorton: species not stated - "*Tantalia*" (18 characters); **Okada**: species not stated - "*Scaptomyza*" (14 characters); **Grimaldi**: *albovittata* (218 characters); **Thomas** and **Hunt**: *albovittata* (*Adh*: M80925); **DeSalle**: *albovittata* (*16S*: XXX*; *COII*: XXX*).

Specimen Information: Representatives of *S. anomala* were obtained from the National *Drosophilia* Species Stock Center (33000-2661.0).

Zapriomus

Throckmorton: species not stated - "*Zapriomus*" (18 characters); **Okada**: species not stated - "*Zapriomus*" (14 characters); **Grimaldi**: *vittiger* (218 characters); **DeSalle**: *tuberculatus* (*16S*: XXX); **Maryuama** and **Hartl**: *tuberculatus* (*Adh*: X63955); **Pelendakis**: tuberculatus (28S: XXX); **Kwiatowski**: *tuberculatus* (*Gpdh*: XXX; *Sod*: XXX); **O'Grady**: *tuberculatus* (*COII*: XXX*).

Specimen Information: Representatives of *Z. tuberculatus* were obtained from the National *Drosophilia* Species Stock Center (50000-2741.0).

Zygothrica

Throckmorton: species not stated - "*Zygothrica*" (18 characters); **Okada**: species not stated - "*Zygothrica*" (14 characters); **Grimaldi**: *prodispar* (218 characters); **O'Grady**: *sp 183* (*16S*: XXX*; *COII*: XXX*).

Specimen Information: **O'Grady**: Estacion Biological La Selva, Costa Rica; 22 February-1 March 1997 (O10.5 and others). Approximately one hundred individuals were collected by sweeping over or aspirating from several fungal blooms.

Ceratitis

Kwiatowski: *capitata* (*Gpdh*: XXX; *Sod*: XXX)

Chloropidae

O'Grady: *sp* (*COII*: XXX*)

Ephydridae: (*Scatella*)

O'Grady: *stagnalis* (*COII*: XXX*)

Ephydridae: ()

O'Grady: *stagnalis* (*COII*: XXX*)

Ephydridae: ()

O'Grady: *stagnalis* (*COII*: XXX*)

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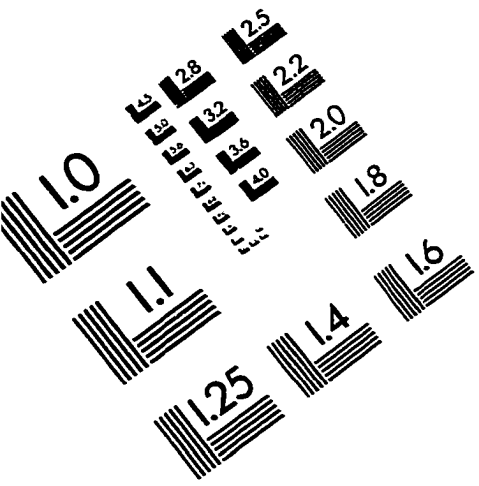
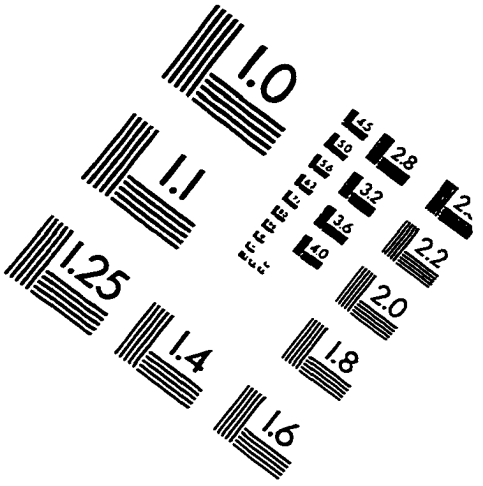
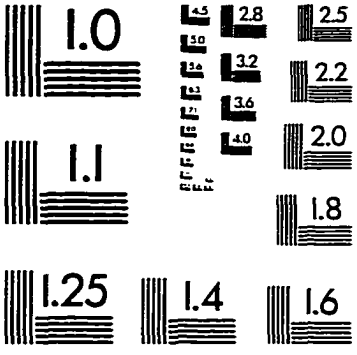
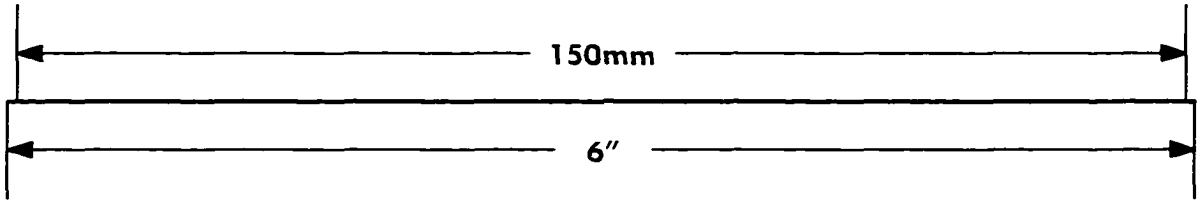
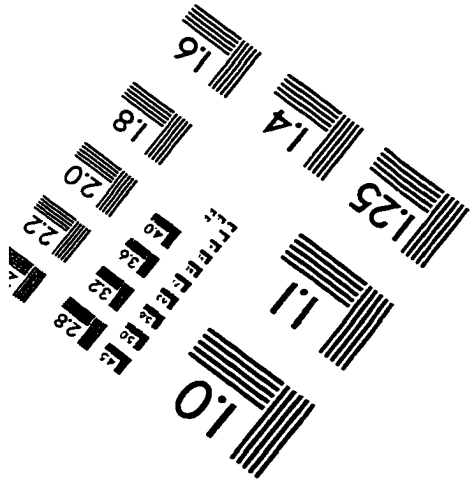
IMAGE EVALUATION TEST TARGET (QA-3)

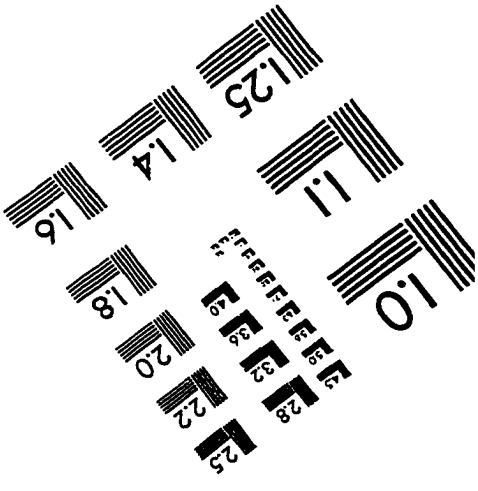












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