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Reevaluation of Phylogeny in the *Drosophila obscura* Species Group Based on Combined Analysis of Nucleotide Sequences

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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. A phylogeny of the *obscura* species group based on combined analysis of nucleotide sequences from six mitochondrial and five nuclear loci is presented here. 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. © 1999 Academic Press

***Key Words: Drosophila obscura* species group; phylogeny; simultaneous analysis.**

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 were 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) summarized these data and indicated that the *obscura* subgroup can be divided into two subgroups, *obscura* and *pseudoobscura* (Fig. 1B). Several phylogenetic analyses have shown that the *pseudoobscura* subgroup, which is distributed exclusively in the New World, is 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 mitochondrial (mt) (Beckenbach *et al.*, 1993; Barrio *et al.*, 1994; Gleason *et al.*, 1997) and five nuclear (nu) loci (Ruttkay

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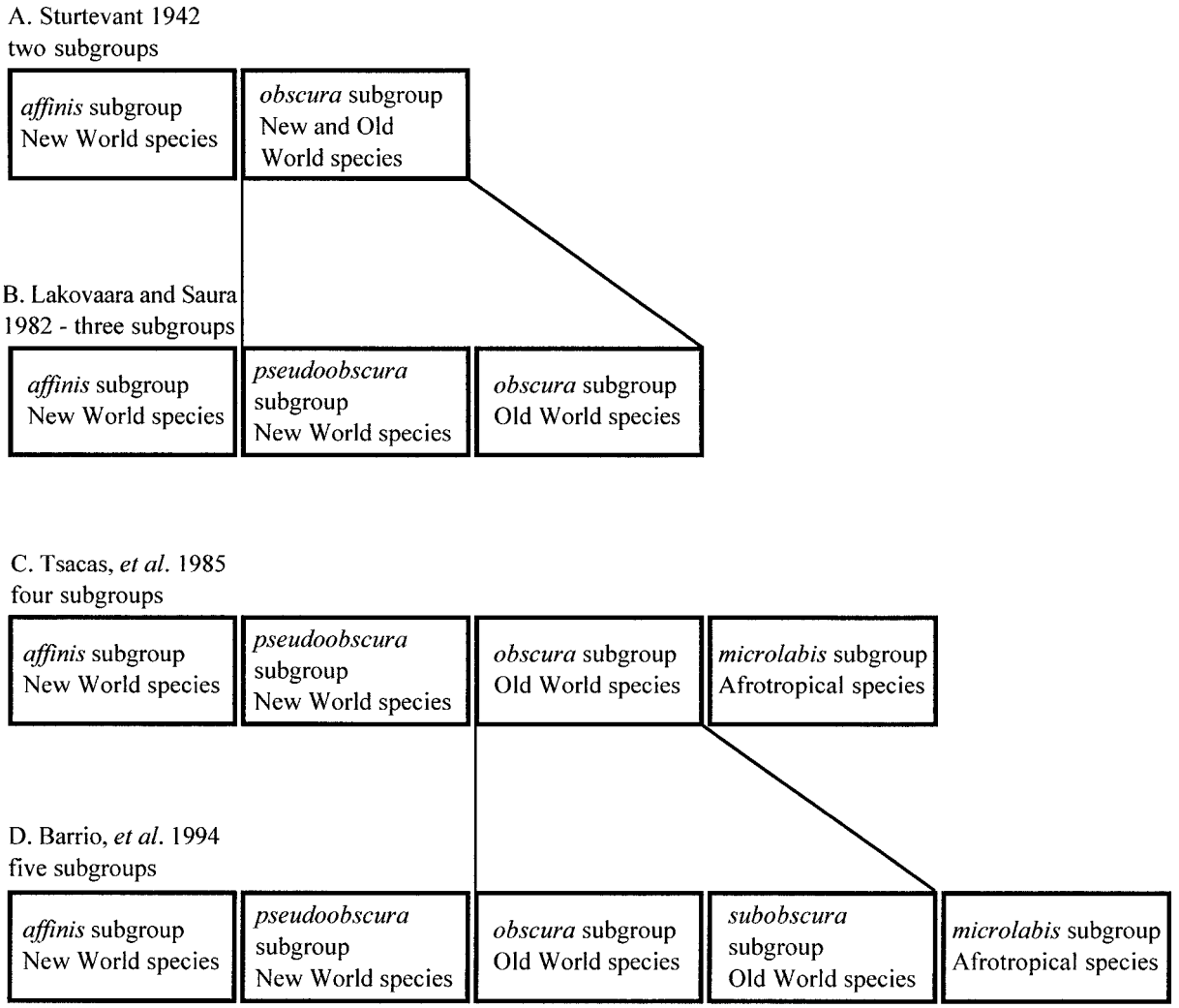


FIG. 1. Taxonomic history of the five subgroups in the *Drosophila obscura* species group, with references.

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, OH (*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

TABLE 1

Taxonomy of the *Drosophila obscura* Species Group with Abbreviations Used in This Study

affinis Subgroup	*pseudoobscura* Subgroup
D. affinis—AFF	*D. lowei*—LOW
D. algonquin—ALG	*D. miranda*—MIR
D. athabasca—ATH	*D. persimilis*—PER
D. azteca—AZT	*D. pseudoobscura*—PSE
D. narragansett—NAR	*D. pseudoobscura "bogotana"*—PBO
D. tolteca—TOL	*obscura* Subgroup
microlabis Subgroup	*D. ambigua*—AMB
D. kitumensis—KIT	*D. bifasciata*—BIF
D. microlabis—MIC	*D. imaii*—IMA
subobscura Subgroup	*D. obscura*—OBS
D. guanche—GUA	*D. subsilvestris*—SSI
D. madeirensis—MAD	*D. tristis*—TRI
D. subobscura—SOB	

Gloor and Engels (1992). Each locus was amplified from the four species in this study using standard PCR cycling conditions. Oligonucleotides 4682 5′ ACATYCAGCCAIGAGTTGAAYTTGTG 3′, located in the first exon, and 4683 5′ CTGGGIGGCATTGGIYTSGACACCAC 3′, located in the third exon, were used to amplify portions of the second exon of the alcohol dehydrogenase (*Adh*) gene from *D. tolteca*. PCR products from the *Adh* gene were then cloned into the TA cloning vector (Invitrogen). Two colonies were selected and a dsDNA cycle sequencing procedure (GIBCO-BRL) was employed to determine the nucleotide sequence of one strand from each clone. Where discrepancies existed between clones of the same species, the differences were verified by consulting the original autoradiograms. 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) 5′ ATGGCAGATTAGTGCAATGG 3′ and (2) 5′ GTTTAAGAGACCAGTACTTG 3′. 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 was 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

TABLE 2

Genbank Accession Numbers of the *Drosophila obscura* Group Sequences Used in This Study

	COI[a]	*COII*[b]	*cytb*[c]	*ND5*[c]	*16S*[c]	*ND1*[c]	*Sod*[d]	*Gpd*[e]	*Adh*[f]	*5S*[g]	*28S*[h]
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	AF081357	—	—
KIT	—	—	—	—	—	—	—	—	—	—	*
MIC	—	—	—	—	—	—	—	—	—	—	*
LOW	—	M95142	—	—	—	—	—	—	—	—	—
MIR	U51608	M95148	U07317	U07316	U07319	U07318	U47870	U47882	M60998	—	—
PER	U51609	M95143	U07327	U07324	U07329	U07328	U47873	U47886	M60997	—	*
PSE	U51606	M95145	—	—	—	—	U47871	U47885	X68164	U58691	X71203
PBO	U51607	—	U07321	U07320	U07323	U07322	U47872	U47891	M60994	—	—
AMB	U51610	M95150	U07297	U07296	U07299	U07298	U47868	U47880	X54813	U58687	*
BIF	U51611	M95147	U07313	U07312	U07315	U07314	U47869	U47883	U40986	U58694	*
GUA	U51612	AF081354	U07326	U07294	U07295	U07294	U47889	U47878	X60113	U58703	*
IMA	—	—	—	—	—	—	—	—	U40987	U58692	—
MAD	U51613	AF081355	U07325	U07290	U07292	U07291	U47887	U47890	X60112	U58710	—
OBS	U51614	AF081356	U07301	U07300	U07303	U07302	U47892	U47881	—	U58715	*
SOB	U51615	M95151	U07287	U07286	U07289	U07288	U47888	U47877	M55545	U58721	*
SSI	U51616	—	U07309	U07308	U07311	U07310	—	U47884	—	U58695	—
TRI	U51617	—	U07305	U07304	U07307	U07306	—	—	—	U58707	*
MEL[i]	U51619	J01404	J01404	J01404	J01404	J01404	X13780	X14179	M17833	—	X71167
YAK[i]	X03240	X03240	X03240	X03240	X03240	X03240	—	—	X54120	—	X71159

[a] *COI* sequences are from the following studies: all ingroup species and MEL = Gleason *et al.* (1997); YAK = Clary and Wolstenholme (1985).

[b] *COII* sequences are from the following studies: GUA, MAD, and OBS = present study; all other ingroup species = Beckenbach *et al.* (1993); MEL = de Bruijn (1983); YAK = Clary and Wolstenholme (1985).

[c] The *cytb*, *ND5*, *ND1*, and *16S* sequences are from the following studies: all ingroup species = Barrio *et al.* (1994); MEL = de Bruijn (1983); YAK = Clary and Wolstenholme (1985).

[d] *Sod* sequences are from the following studies: all ingroup species = Barrio and Ayala (1997); MEL = Kwiatowski *et al.* (1989).

[e] *Gpdh* sequences are from the following studies: all ingroup species = Barrio and Ayala (1997); MEL = Bewley *et al.* (1989).

[f] *Adh* sequences are from the following studies: AMB = Marfany and Gonzalez-Duarte (1991a); GUA and MAD = Marfany and Gonzalez-Duarte (1993); SOB = Marfany and Gonzalez-Duarte (1991b); TOL = this study; MIR, PBO, and PER = Schaeffer and Miller (1991); PSE = Schaeffer and Miller (1992); MEL = Kreitman (1983); YAK = Ashburner (unpublished).

[g] *5S* sequences are from Grau and Bachmann (1997).

[h] *28S* sequences are from the following studies: AFF, AZT, PSE, MEL, and YAK = Pelendakis and Solignac (1993); KIT, MIC, PER, AMB, BIF, GUA, OBS, SOB, and TRI = Ruttkay *et al.* (1992).

[i] Outgroup sequences. MEL = *Drosophila melanogaster* and YAK = *Drosophila yakuba.*

* Sequences not present in GenBank.

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). At least one nucleotide sequence from four of the five currently recognized *obscura* subgroups was determined for all sequences analyzed in the individual analyses. The *5S* locus was not examined individually because there were no alignable outgroup sequences determined.

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. The *5S* locus was included in these analyses because, even though there was no outgroup present, characters were able to resolve some clades in the ingroup. 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 a 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). It has been suggested that concordance of divergent phylogenetic reconstruction methods is a way to improve the accuracy of phylogenetic estimation (Kim, 1993); so several methods were compared and contrasted in this study (Table 3). Individual MP searches were performed with the branch and bound algorithm (PAUP 4.0; Swofford, 1998). 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. Neighbor joining (NJ) analyses were performed in PAUP*4.0 (Swofford, 1998) using a Kimura two-parameter model. Support was assessed using bootstrap proportions (500 replicates). Individual ML analyses were performed as follows: (1) the transition–tranversion ratio, proportion of invariant sites, and γ shape parameter were estimated for the most parsimonious tree(s). When more than one equally parsimonious tree was present, the parameters from the tree with the best $-\ln$ likelihood score were used. (2) The parameter values estimated above were used in a likelihood search (search type = heuristic, starting trees obtained by random addition, replicates = 100, TBR branch swapping) using PAUP*4.0 (Swofford, 1998). 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 with the *melanogaster* species group, either *D. melanogaster* or *D. yakuba* or both. Figures 2 and 3 shown the results of individual MP analyses. Differences in topology between MP, NJ, and ML trees are described in Table 3.

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 the 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. Pairwise tests were performed to look for incongruence between individual partitions (Table 4). When comparing individual loci with the PHT, data sets were trimmed to contain only those taxa in common between the two partitions. A PHT was also performed 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 4).

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). *D. melanogaster* and *D. yakuba* were outgroups in combined analyses, as described above. Support for each node was determined as above. Partitioned Bremer support (PBS; Baker and DeSalle, 1997) was also used 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). 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 indicate the level of support for, or incongruence with, a node. The sum of all partition lengths for any

TABLE 3

Comparisons between Individual MP, ML, and NJ Analyses

Locus	Method	Topology[a]	Model employed[b]
COII	MP	Fig. 2A	Unweighted
	NJ	(1) TOL–NAR unresolved (2) BIF–*subobscura* (BP = 53)	Kimura two-parameter
	ML	(1) TOL–NAR unresolved (2) BIF–*subobscura* (BP = 61) (3) AMB–OBS–*affinis*–*pseudoobscura* (BP = 57)	ti/tv = 2.523 Prop. invar. = 0.655 γ = 2.185
cytb	MP	Fig. 2B	Unweighted
	NJ	Identical to MP tree	Kimura two-parameter
	ML	MAD–SOB (BP = 65)	ti/tv = 2.352 Prop. invar. 0.678 γ = 2.244
16S	MP	Fig. 2C	Unweighted
	NJ	(1) AFF–ALG–ATH (BP = 54) (2) *affinis–pseudoobscura–subobscura* (BP = 57) (3) AMB–OBS (BP = 61) (4) AMB–OBS–BIF (BP = 53) (5) SSI–TRI (BP = 91)	Kimura two-parameter
	ML	(1) AFF–ALG–ATH (BP = 62) (2) AMB–BIF–OBS (BP = 60)	ti/tv = 0.436 Prop. invar. = 0.699 γ = 0.663
ND5	MP	Fig. 2D	Unweighted
	NJ	(1) BIF–*affinis* (BP = 68) (2) MAD–SOB (BP = 79)	Kimura two-parameter
	ML	(1) BIF–*affinis* (BP = 69) (2) *affinis–pseudoobscura–obscura* (BP = 54)	ti/tv = 2.202 prop. invar. = 0.596 γ = 0.772
ND1	MP	Fig. 2E	Unweighted
	NJ	(1) AMB–OBS–TRI–SSI (BP = 58)	Kimura two-parameter
	ML	(1) AMB–OBS–TRI–SSI (BP = 57)	ti/tv = 2.182 Prop. invar. = 0.745 γ = 1.478
COI	MP	Fig. 2F	Unweighted
	NJ	Identical to MP tree	Kimura two-parameter
	ML	(1) AMB–OBS–TRI (BP = 56) (2) *affinis–obscura–subobscura* (BP = 53)	ti/tv = 1.699 Prop. invar. = 0.672 γ = 2.948
Sod	MP	Fig. 3A	Unweighted
	NJ	(1) AMB–OBS (BP = 100)	Kimura two-parameter
	ML	Identical to MP tree	ti/tv = 1.192 Prop. invar. = 0.485 γ = infinity (300)
Gpdh	MP	Fig. 3B	Unweighted
	NJ	(1) *affinis* (BP = 73) (2) *subobscura–pseudoobscura–obscura* clade (BP = 65) (3) AZT–TOL (BP = 51)	Kimura two-parameter
	ML	Topology is identical to MP tree	ti/tv = 2.03 Prop. invar. = 0.670 γ = infinity (300)
Adh	MP	Fig. 3C	Unweighted
	NJ	(1) *pseudoobscura–subobscura–obscura* (BP = 78) (2) BIF–IMA–*subobscura* (BP = 74)	Kimura two-parameter
	ML	Topology identical to MP tree	ti/tv = 1.521 Prop. invar. 0.664 γ = 8.334
28S	MP	Fig. 3D	Unweighted
	NJ	Topology identical to MP tree	Kimura two-parameter
	ML	Topology identical to MP tree	ti/tv = 1.098 Prop. invar. = 0 γ = 2.188

[a] If the NJ or ML tree depicts a relationship different from that of the MP, the species (as Table 1) and species subgroup forming the clade are shown.

[b] All MP searches were performed unweighted. All NJ analyses used the Kimura two-parameter model of substitution. The following parameters were estimated on the MP tree(s) for use in the ML analyses: transition/transversion (ti/tv) ratio, proportion of invariant sites (prop. invar.), and a γ distribution.

given node will always equal the decay index for that node on the total-evidence tree. This method determines 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. Important aspects of each analysis include the total number of characters and total number of parsimony-informative characters in each locus, the number and length of most parsimonious trees (MPTs) recovered by each search, and the consistency and retention indices for each MPT (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

TABLE 4

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

Note. Numbers shown are *P* values from the Partition Homogeneity Test implemented PAUP*4.
* PHT is significant at the $P < 0.1$ level.

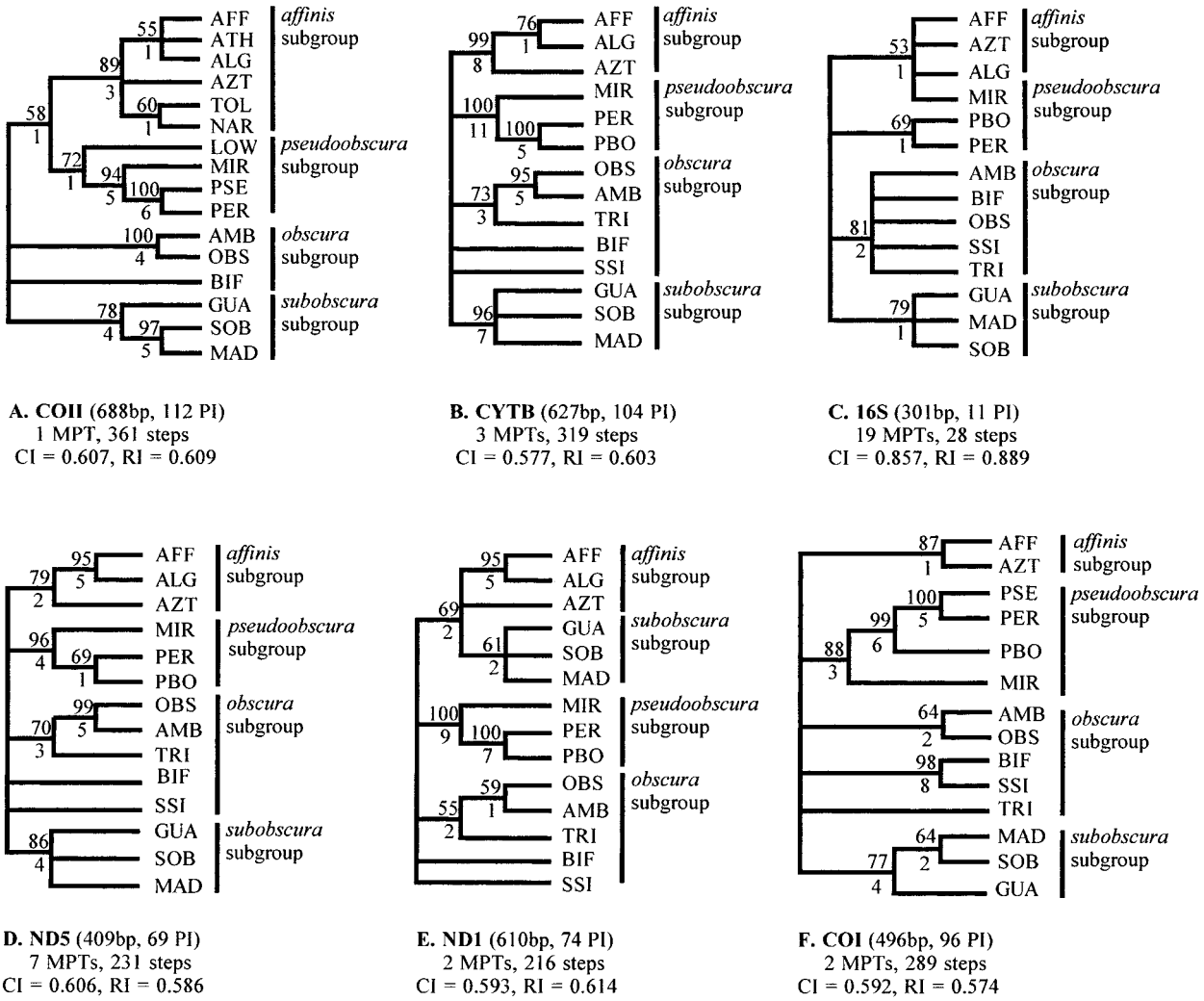


FIG. 2. Results of individual analyses of mt DNA sequences. Each phylogeny is shown with the length in base pairs (bp) of the partition, the number of parsimony-informative (PI) characters in the data set, the number of MPTs found, the number of steps on each MPT, and the ensemble consistency and retention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

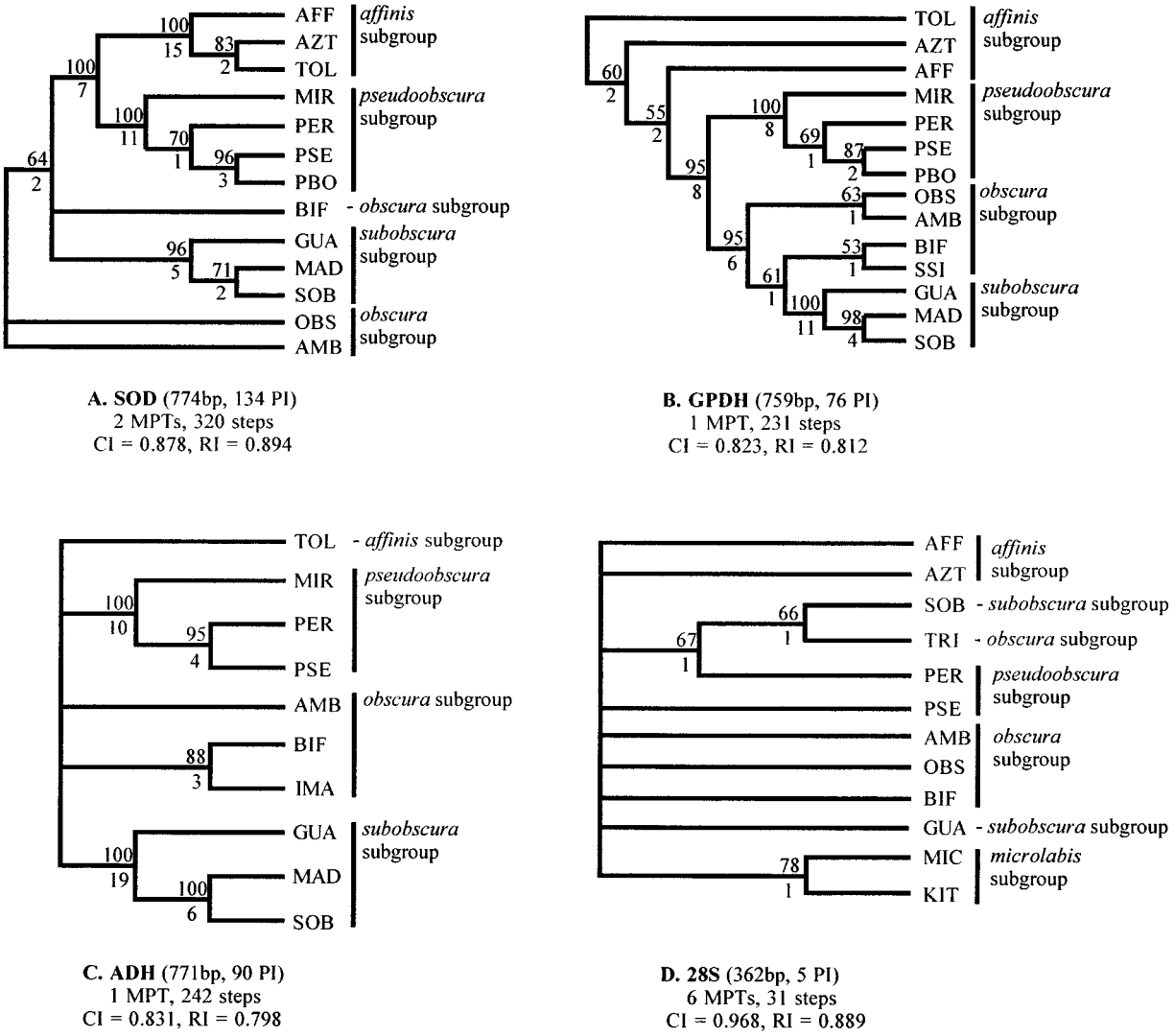


FIG. 3. Results of individual analyses of nu DNA sequences. Each phylogeny is shown with the length in bp of the partition, the number of parsimony-informative characters in the data set, the number of MPTs found, the number of steps on each MPT, and the CI and RI values. BPs are shown above and DIs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

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 the sister group (or groups) of 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 met *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 nonmonophyletic 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 four of the five *obscura* subgroups. This partition gives results similar to those of 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 nonmonophyletic.

Comparison between Phylogenetic Methods

Table 3 lists the differences in topology of the ML and NJ analyses, relative to the individual MP analyses presented in Figs. 2 and 3. Sometimes the results of an analysis based on one phylogenetic method will support relationships which are not seen in analyses based on other methods. In the individual *Gpdh* analysis, for example, MP and ML analyses indicate that the *affinis* subgroup is not monophyletic. The NJ study, however, recovers a monophyletic *affinis* subgroup, a more conventional result. In this case it may be that the Kimura two-parameter model employed by NJ is better in matching the model of evolution of the *Gpdh* sequence than are the other two methods.

When a node exists in the NJ or ML tree that is absent in the MP tree, support for that node is usually weak (BP $<$ 65). There are, however, several instances when a highly supported node is present in the NJ or ML tree, but not seen in the MP tree. In one NJ analysis, the SSI–TRI clade is supported with a BP = 91, but this node is unresolved in the MP strict consensus tree. This is because the NJ search found only a single tree and the MP analysis was able to find 11 equally parsimonious trees, one of which had a SSI–TRI clade. The results of the MP, NJ, and ML analyses broadly agree with one another. Only the MP analyses are summarized below to facilitate comparisons to the combined analyses, which employed only the MP criterion.

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), was 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 4). The four nu loci compared are incongruent in four comparisons (Table 4). 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 being incongruent with only one mt locus (*COI* and *16S*, respectively; Table 4). The nu *Sod* gene is incongruent with all mt partitions except for *COII* (Table 4). The nu *Gpdh* partition is congruent with only the mt *COII* and *cytb* genes (Table 4). 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

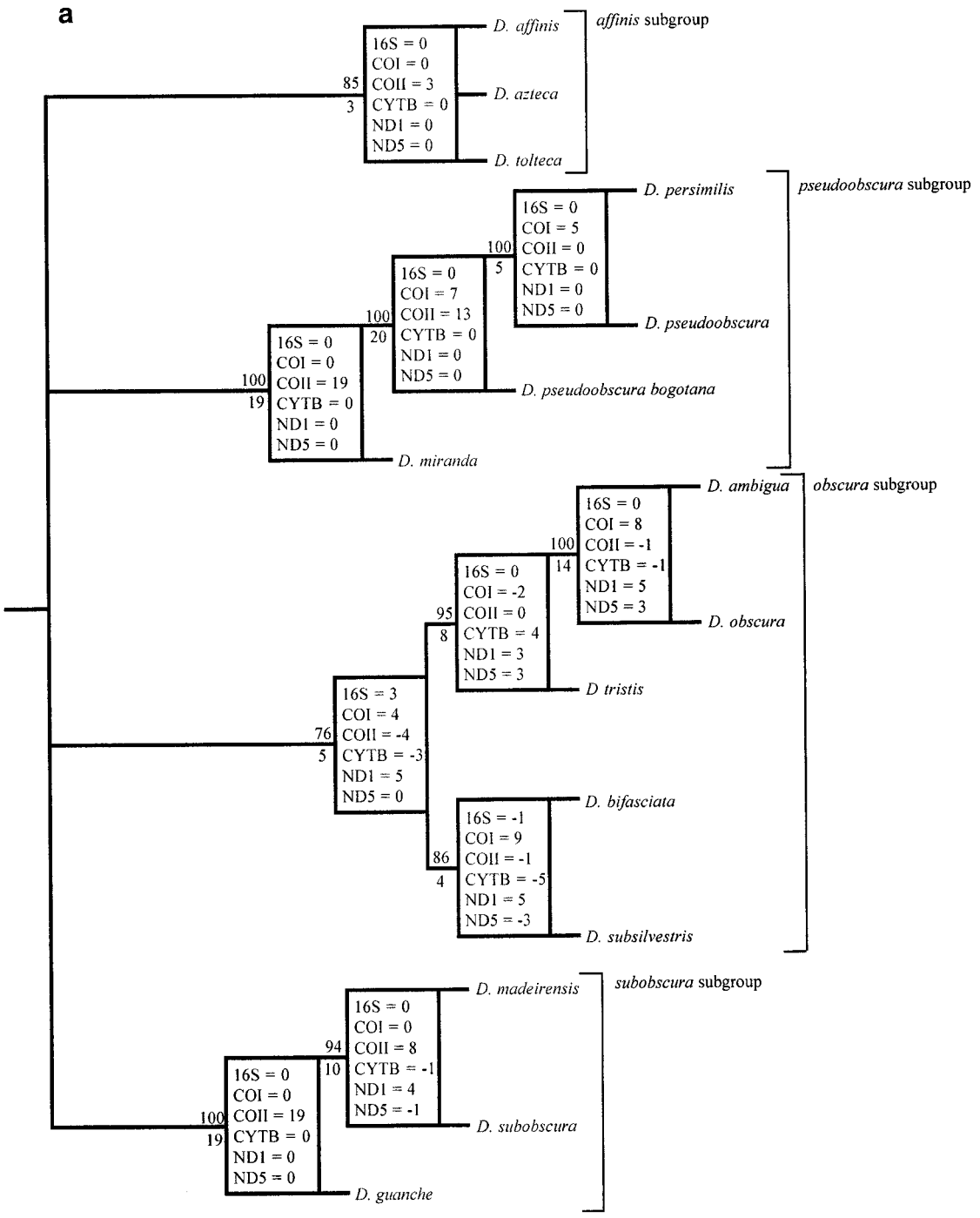
The individual data partitions were then combined into two data sets, nu and mt, to determine if different classes of data (i.e., nonrecombining, maternally inherited vs recombining, biparentally inherited) yield different topologies when analyzed (Figs. 4A and 4B) or are significantly incongruent with 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; BP = 76, DI = 5; and BP = 100, DI = 19; respectively), although these data are 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 = 100, DI = 24; 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 due to either a lack of resolution or the shifting of relationships between closely related species. An example of topological conflict between closely related species is found in the *p. pseudoobscura–persimilis–p. bogotana* clade. The mt DNA partitions (Fig. 4A) indicate that *D. persimilis* and *D. p. pseudoobscura* are sister taxa to the exclusion of *D. p. bogotana*. This agrees with the combined mt analysis of Gleason *et al.* (1997), but is in conflict with the nu analyses, which indicate that the subspecies of *D. pseudoobscura* are sister taxa (Fig. 4B). Such a conflict between nu and mt characters may be the result of an introgression of mtDNA, but not nuDNA, from *D. persimilis* to *D. p. pseudoobscura*. It is also possible, however, that this is an artifact of species sampling differences among the various studies represented in this data set. Of the mtDNA studies, only the *COI* study examined *D. persimilis*, *D. p. pseudoobscura*, and *D. p. bogotana*. The other data sets used only one subspecies, either *D. p. pseudoobscura* or *D. p. bogotana*, to represent *D. pseudoobscura* (Table 2). Therefore, the only mt characters which resolve relationships among these taxa come from *COI*, which may be saturated for transitional changes (Gleason *et al.*, 1997). Two nuDNA data sets, *Gpdh* and *Sod*, sampled all three taxa (Table 2) and the results of these analyses, both individually (Figs. 3A and 3B) and combined (Fig. 4B), support the *p. pseudoobscura–p. bogotana* sister group relationship. Inclusion of missing *D. pseudoobscura* subspecies representatives in the nu and mt DNA data sets may be able to determine whether the topological differences in Figs. 4A and 4B are due to introgression or sampling artifacts. A partition homogeneity test indicates that the nu and mt data partitions are not significantly heterogeneous with respect to one another (Table 4); so these data were combined and analyzed simultaneously (Fig. 5).

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

FIG. 4. (A) Results of combined mitochondrial analysis. (B) Results of combined nuclear analysis. Each phylogeny is shown with the length in bp 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.



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

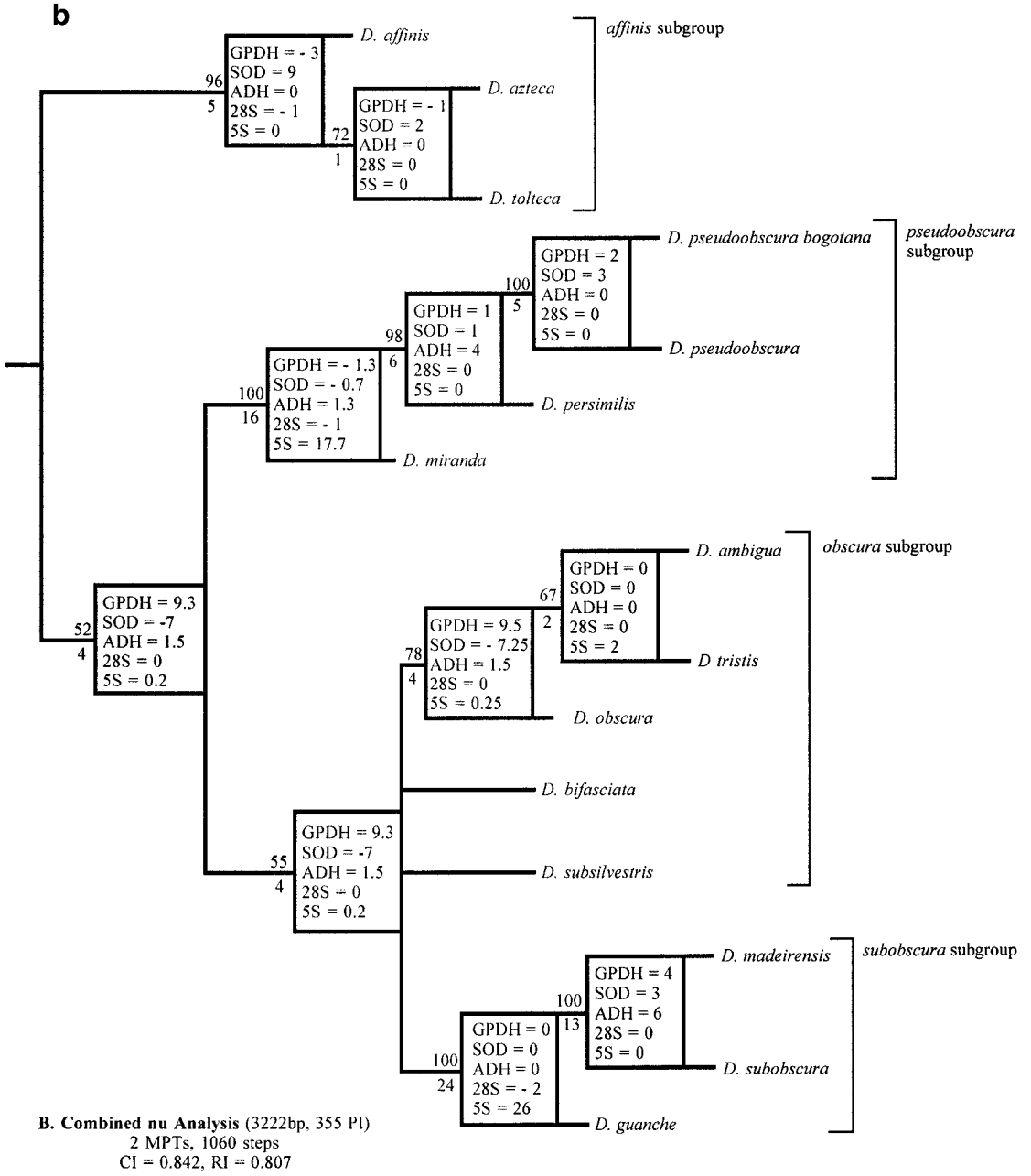


FIG. 4—*Continued*

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 a negative summed PBS (−3.95), it is congruent with (i.e., has either a positive or a zero 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 4B). 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 (e.g., 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. Cunningham (1997) has suggested that, when misleading data are overcome by the inclusion of additional characters, combining incongruent data sets may actually improve phylogenetic estimation. Baker and DeSalle (1997) also argue for the combination of incongruent data, but on the basis that although two data sets are significantly incongruent at a certain node in the tree, they may also agree strongly at several other nodes.

The nucleotide sequence data for the *D. obscura* group offer a unique opportunity to examine the conditional combination approach to phylogenetic analysis. Several partitions can be constructed for comparison using the data currently available, including nuclear vs mitochondrial loci, protein coding vs ribosomal loci, stems vs loops within ribosomal sequences, and codon position within protein coding sequences. This study compares the mitochondrial partitions (Beckenbach *et al.,* 1993; Barrio *et al.,* 1994; Gleason *et al.,* 1997) to the nuclear partitions (Ruttkay *et al.,* 1992; Russo *et al.,* 1995; Barrio and Ayala, 1997; Grau and Bachman, 1997) in their ability to reconstruct the phylogeny of the *D. obscura* group.

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 4). 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, may not preclude data set combination.

The PBS values that are presented for each node in the combined analyses indicate that each gene partition contributes, either positively or negatively, to the topology of simultaneous analysis trees (Figs. 4 and 5). Many of those loci which have high positive summed PBS values also conflict (i.e., have negative PBS val-

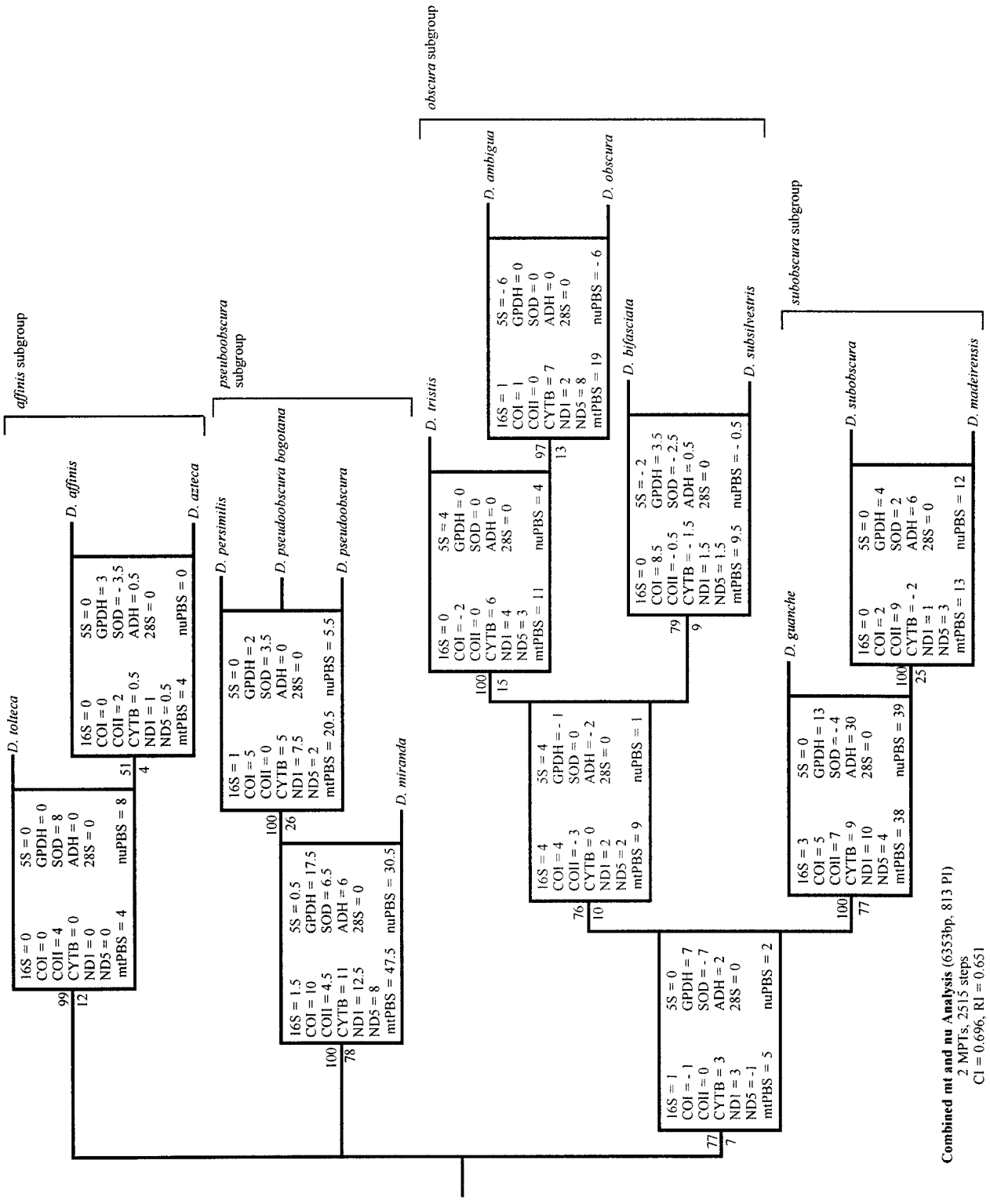


FIG. 5. Results of combined analysis 2. Each phylogeny is shown with the length in bp of the partition, the number of parsimony-informative characters in the data set, the number of MPTs found, the number of steps on each MPT, and the CI and RI values. BPs (above node), DIs (below node), and PBS values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

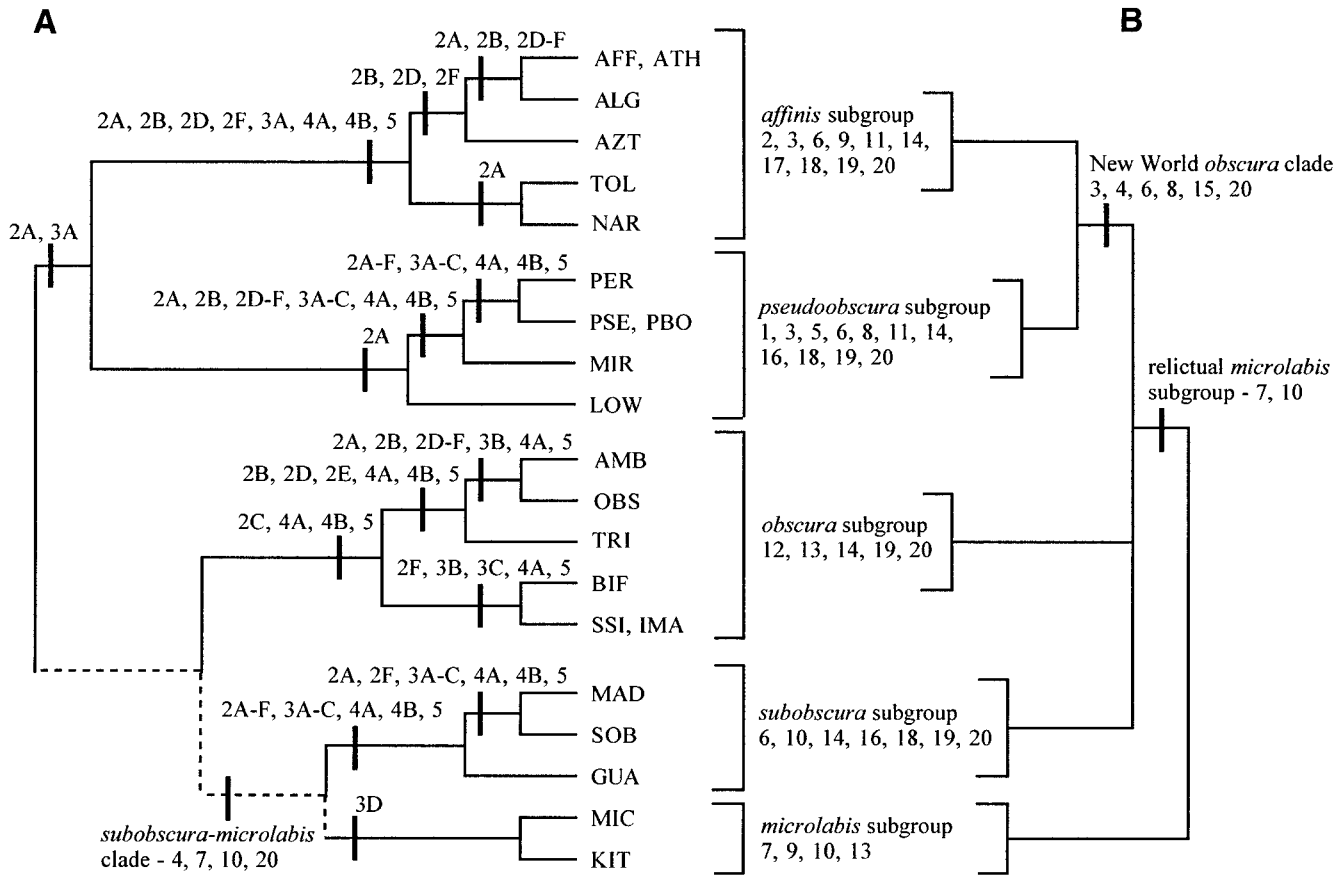


FIG. 6. Summary diagram showing two possible phylogenetic hypotheses for the *D. obscura* species group. (A) Phylogenetic relationships supported by individual and combined analyses in this study. (B) Phylogenetic relationships proposed by Gleason *et al.* (1997) based on analysis of mtDNA sequences. Numbers of the Figures in this paper supporting each node are 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.

ues) at some nodes. For example, *Gpdh* is either negative or zero at 4 of the 11 nodes, even though it contributes the highest summed PBS of all loci in the tree. Based upon these results, it may 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 in this study effectively track historical relationships at one taxonomic level, while contributing to incongruence at another. Combining all the data, even if it is incongruent, into one unweighted simultaneous analysis is perhaps preferable. Weighting or combining only congruent data sets can result in the omission of many characters, 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.

Phylogeny of the D. 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 morphology, polytene chromosome banding patterns, allozymes, restriction fragment length polymorphisms, DNA–DNA hybridization, 1- and 2-D gel electrophoresis, and nucleotide sequences, have gradually refined the 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 among 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 in which this

group is thought to have formed (Throckmorton, 1975; Gleason *et al.*, 1997).

Figure 6 presents two possible 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 one 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 (Gleason *et al.*, 1997; Fig. 6B).

The present study proposes that the Afrotropical *microlabis* subgroup forms a clade with the *subobscura* subgroup, which has a distribution that 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, unpublished). 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).

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, is clearly required in future studies. 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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