

Phylogenetic Utility of the Mitochondrial Cytochrome Oxidase Gene: Molecular Evolution of the *Drosophila buzzatii* Species Complex

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Abstract. Phylogenetic relationships among eight species of the *Drosophila buzzatii* species complex (*D. mulleri* subgroup; *D. repleta* species group) and *D. hamatofila* were determined by sequencing the mitochondrial cytochrome oxidase subunit I, II, and III genes. The species examined included members of the *martensis* cluster (*D. martensis*, *D. starmeri*, *D. venezolana*), the *buzzatii* cluster (*D. buzzatii*, *D. serido*, *D. borborema*), and the *stalker* cluster (*D. stalker*, *D. richardsoni*). The molecular phylogeny was found to be congruent with the chromosomal inversion phylogeny. Analyzing the cytochrome oxidase subunits separately revealed that not all the subunits seem to have the same phylogenetic information content. Parameters are discussed that might explain these differences.

Key words: *Drosophila* systematics — DNA sequence variation — Base composition bias — Maximum likelihood — Γ -distributed rates model

Introduction

The *Drosophila buzzatii* complex is one of several complexes that comprise the *D. mulleri* subgroup, which is part of the *D. repleta* species group. This complex was recently designated by Ruiz and Wasserman (1993), based on some new chromosomal inversion and inter-specific hybridization data (Marin et al. 1994). They de-

finied the *buzzatii* complex as consisting of the *buzzatii* cluster (*D. buzzatii*, *D. serido*, *D. borborema*, *D. koepferae*), the *martensis* cluster (*D. martensis*, *D. uniseti*, *D. starmeri*, *D. venezolana*), and the *stalker* cluster (*D. stalker*, *D. richardsoni*). Since their study, the species *D. seriema* has been described as a member of the *buzzatii* cluster (Tido-Sklorz and Sene 1995). Both the *martensis* and *buzzatii* clusters are predominantly South American in distribution (except for *D. buzzatii* which is now subcosmopolitan), with the *stalker* cluster restricted to Florida and the Caribbean. They are all cactus feeding flies as far as is known, although very little work has yet been done on their ecology (Sene et al. 1982; Pereira et al. 1983; Benado et al. 1984).

The revised chromosomal phylogeny (Fig. 1) is different from even recently proposed versions of the evolutionary relationships (e.g., Wasserman 1992). This is due to a more detailed examination of the chromosomal inversion patterns, which established the closeness of the two groups by establishing two inversions that appear to unite the groups (Ruiz and Wasserman 1993). This closeness was confirmed by the observation that the *stalker* complex could hybridize with some species of the *buzzatii* and *martensis* clusters (Marin et al. 1994). Previous classifications had already placed the *martensis* and *buzzatii* clusters together (Wasserman 1982a,b), but no association with the *stalker* cluster had been suggested. In fact, the *stalker* cluster was until recently considered a complex with unclear affinities to any other groups in the *D. mulleri* subgroup (Wasserman 1992).

Now, with a detailed chromosomal inversion phylogeny, it is possible to compare the evolutionary history of the group from a molecular perspective and determine

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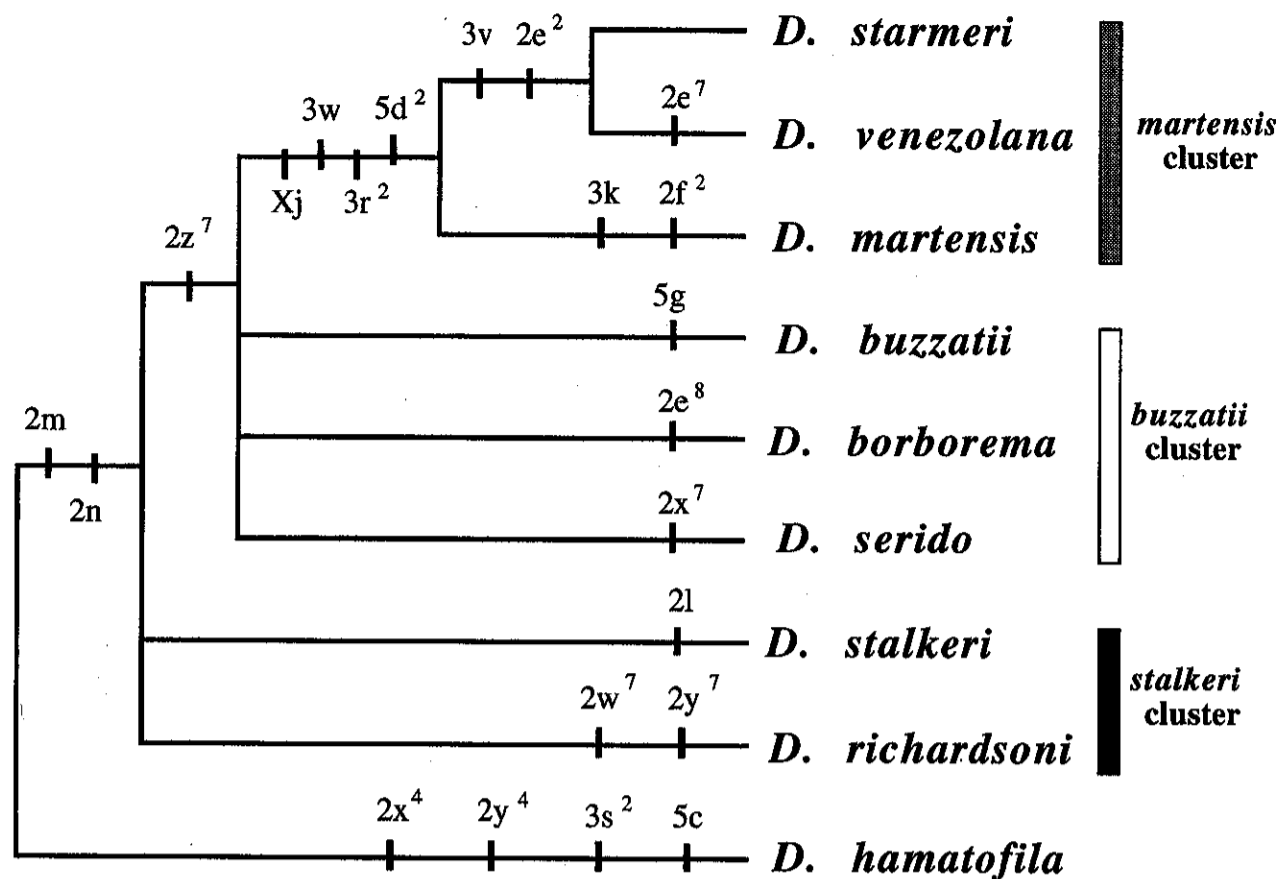


Fig. 1. Chromosomal inversion phylogeny and classification of the *D. buzzatii* species complex according to Ruiz and Wasserman (1993). Only fixed interspecific differences are shown.

the amount of congruence between the two data sets. Previous studies have confirmed the congruence of chromosomal inversion and molecular data sets among *Drosophila* species (MacIntyre and Collier 1986; Spicer 1992). In an effort to examine the congruence between a phylogeny derived from a protein-coding mitochondrial gene and an established chromosomal phylogeny, the phylogenetic relationships among the eight available species in the *D. buzzatii* complex and *D. hamatofila* were examined by sequencing 1,515 base pairs of the mitochondrial DNA-encoded (mtDNA) cytochrome oxidase subunits I, II, and III (COI, II, III). Moreover, this presents an opportunity to examine questions concerning the combination molecular data sets (Cracraft and Helm-Bychowski 1991; Olmstead and Sweere 1994), the amount of sequence necessary for accurate phylogenetic reconstruction and genetic distance estimates (Martin et al. 1990), and other parameters that could cause some polypeptide chains and the corresponding genes to be superior to others for phylogenetic analysis.

Materials and Methods

The DNA sequences of the mitochondrial cytochrome oxidase I, II, and III subunits from nine species of the *D. mulleri* subgroup were obtained by the direct sequencing of PCR products. The corresponding mtDNA sequences from *D. yakuba* and *D. melanogaster*, which were used as outgroups in this study, came from Clary and Wolstenhome (1985) and de Bruijn (1983), respectively.

Primers. The oligonucleotides that were used for PCR amplification and sequencing are listed in Table 1. The amplification primers for COI were initially developed in R. Harrison's laboratory at Cornell University, but these have been made specific to *Drosophila*. Most of the COII primers appeared in Liu and Beckenbach (1992), although

some have modified from their original compilation. The amplification primers for COIII were designed by C. Simon and C. Oregó and appeared in Simon et al. (1991). Most of the internal sequencing primers were designed independently.

Specimens. The strains that were used, and their corresponding National *Drosophila* Species Resource Center stock numbers are as follows: *Drosophila martensisi* (15081-1321.0), Barquisimeto, Venezuela; (15081-1321.1), Barcelona, Venezuela; *D. borborema* (15081-1281.0), Cafarnaum, Morro do Chapen, Bahia, Brazil; (15081-1281.2), Cafarnaum, Morro do Chapen, Bahia, Brazil; *D. serido* (15081-1431.1), Rio Paraguassa, Aegoon, Bahia, Brazil; (15081-1431.3), Milagres, Bahia, Brazil; *D. buzzatii* (15081-1291.0), Cochabamba, Bolivia; *D. starkeri* (15081-1461.0), Puerto Cabello, Venezuela; *D. venezolana* (15081-1496.0), La Esmeralda, Venezuela; *D. starkeri* (15081-1451.2), Cayman Brac, Cayman Islands; *D. richardsoni* (15081-1421.1), Montserrat Airport; *D. hamatofila* (15081-1301.0), Tucson, Arizona.

Sample Preparation. Total genomic DNA was isolated by grinding 1-5 flies with a teflon grinding implement. This was performed in a 1.5 ml tube containing 500 μ l of grinding buffer (0.1 M EDTA, 100 mM Tris pH 8.0, 1% SDS, 0.2 M NaCl). Usually live flies were used, but 70% alcohol preserved specimens also have been successfully extracted, amplified, and sequenced. The homogenate was incubated overnight at 65°C, and then extracted with equilibrated phenol several times until the supernatant was not cloudy or discolored. The supernatant was then extracted two times with chloroform, then with cold 100% ethanol, and finally several times with 70% ethanol at room temperature. The DNA was dried and resuspended in 200 μ l of d.d. water.

PCR Amplification. The conditions of the polymerase chain reaction (Mullis et al. 1987; Saiki et al. 1988) were varied depending on the genes being amplified. The primers used for PCR amplification are listed in Table 1. The double-stranded amplifications reaction volumes were usually 50 μ l solutions. Generally, the 5 \times buffer (300 mM Tris-HCL, 75 mM (NH₄)₂SO₄, pH 8.5) was used, along with 10 mM of dNTP's and 10 μ M of the primers. Both the Mg⁺⁺ concentration and pH were adjusted depending on the template. These were varied from a

Table 1. Oligonucleotides used for PCR amplification and sequencing of mitochondrial cytochrome oxidase subunit I, II, and III genes

Primer ^a	Sequence
	Cytochrome oxidase I
C1-J-1751*	5'-GGAGCTCCTGACATAGCATTCCC
C1-J-1847	5'-GGAGCTGGGACAGGGTGAAC
C1-J-2001	5'-ACAAATAAAGGTATTCGGTC
C1-J-2001-s	5'-ACAAATAAAGGTATACGATC
C1-J-2021	5'-GACCGAATACCTTTATTTGT
C1-J-2021-s	5'-GATCGTATACCTTTATTTGT
C1-J-2021-gut	5'-GATCGAATACCATTATTCGT
C1-N-2191*	5'-CCCGGTAAAATTAATAATAAACTTC
	Cytochrome oxidase II
TL2-J-3037*	5'-ATGGCAGATTAGTGCAATGG
C2-J-3279	5'-CAATTAATTGAAATAATTTGAAC
C2-N-3344	5'-GAAGGTTTCAATTTTCATC
C2-J-3402	5'-GGTCATCAATGATATTGAAG
C2-N-3494	5'-GGTAAACTACTCGATTATCAAC
C2-N-3665	5'-CCACAAATTTCTGAACATTG
C2-J-3684	5'-CAATGTTTCAGAAATTTGTGG
TK-N-3785*	5'-GTTTAAGAGACCAGTACTTG
	Cytochrome Oxidase III
C3-J-5014*	5'-TTATTTATTTTATCAGAAGT
C3-J-5131	5'-TTTAATCCATTTCAAATTCC
C3-N-5220	5'-ACTGTAAAAATAATCCTTGAGT
C3-N-5220-buz	5'-ACAGTAAAAATAAACCTTGTGT
C3-J-5242	5'-ACTCAAGGATTATTTTTTACAGT
C3-N-5460*	5'-TCAACAAAATGTCAGTATCA

^a The oligonucleotides are named according to the system established by Simon et al. (1994) for insect mtDNA. The first two characters denote the gene, the next letter establishes the strand, and the numbers indicate the 3' location of the primer for the reference species *Drosophila yakuba* (Clary and Wolstenhome 1985). An asterisk (*) following the name indicates that the primer was used for PCR amplification; oligonucleotide names without an asterisk were only used as sequencing primers. Any other characters following the 3' location number are special designations denoting that a base change(s) was made to produce a species-specific primer

concentration of 7.5–17.5 mM of MgCl₂ and a pH 8.5–9.5 for the buffer. Between 30–35 cycles were used for the amplifications. The denaturing step was set at 94°C for 40 s and the extension step was at 72°C for 1 min. The annealing step varied according to the primers that were being amplified. Usually, this ranged from 48–54°C for 2 min.

Template Purification. The double-stranded templates were purified using the Pharmacia Biotech MicroSpin S-300 HR columns according to the protocol supplied by the manufacturer. In some instances, as for example when an amplification produced more than one band, a more rigorous purification procedure was found to be required. In this case, the double-stranded templates were run on a 2.5% acrylamide gel in a 1× TBE buffer. The acrylamide gel was soaked in an EtBr solution for 30 min, the templates were visualized with a UV light box and then cut from the gel with a razor blade. The gel bands were soaked overnight at 65°C in a 3 M Sodium Acetate solution. The double-stranded DNA product was obtained by spinning the solution three times at 5000 g in a Millipore 30,000 NMWL Ultrafree-MC micro-concentrator.

Sequencing. All direct DNA sequencing of the double-stranded PCR products was performed using the USB Sequenase Kit, but not

according to the manufacturer's guidelines. Instead, a modified protocol was followed (Casanova et al. 1990; Liu and Beckenbach 1992). The denaturing step consists of boiling the template, reaction buffer and primer for 5 min, and then placing this into either a liquid nitrogen or dry ice/ethanol bath. The labeling reaction mixture is added while the sample is still frozen. The reaction mixture was microfuged for 30 s and then added to the extension mix. This was incubated at 37–42°C for 5 min, then terminated.

Sequence Comparisons. A variety of techniques were used to infer the phylogenetic relationships among the taxa. Parsimony analyses were performed by using the computer program PAUP*Star (Swofford 1995). These analyses were accomplished with the branch-and-bound algorithm using both unordered changes and stepmatrices to differentially weight transitions and transversions. When several equally parsimonious trees were found, strict consensus trees (Rohlf 1982) were produced to summarize these data. In order to assess some confidence limits concerning the branching pattern, a bootstrap analysis was performed (Felsenstein 1985). A total of 300 replications was performed using the branch-and-bound algorithm. The result is presented as a majority rule consensus tree (Margush and McMorris 1981), which shows the most frequently occurring branching orders. In addition, to evaluate some alternative less parsimonious arrangements, tree manipulations were accomplished by using the program MacClade (Maddison and Madson 1992). Distance analyses were also performed using PAUP*Star (Swofford 1995) and were clustered using the heuristic search option invoking the minimum evolution method (Saitou and Imanishi 1989). In addition, bootstrap values based on 300 replications were performed. Maximum likelihood was used to evaluate among competing phylogenetic hypotheses inferred by the other techniques and these were carried out by PAUP*Star (Swofford 1995). Finally, the Templeton parsimony test was performed by the DNAPars program in the computer package PHYLIP (Felsenstein 1993).

Results

The sequenced regions of the mitochondrial cytochrome oxidase gene encompass a 408 base pair (bp) segment of subunit I (*D. yakuba* positions 1783–2190; total length of subunit is 1535 bp), the entire subunit II (*D. yakuba* positions 3083–3766) comprising 688 bp, and a 419 bp segment of subunit III (*D. yakuba* positions 5015–5433; total length of subunit is 788 bp). The *D. yakuba* positions refer to the Clary and Wolstenhome (1985) sequence. This results in a total of 1,515 bp sequenced from 12 haplotypes representing nine species.

Table 2 shows the percentage and absolute numbers of variable and phylogenetically informative sites when considering both ingroup taxa, and when including the outgroup taxa (*D. yakuba* and *D. melanogaster*). None of the cytochrome oxidase subunits are more or less variable than another as determined by a Chi-square test. This not only includes the comparison when just considering the ingroup taxa ($\chi^2 = 3.19$, df = 2, P = 0.202), but also when considering the outgroup taxa ($\chi^2 = 3.00$, df = 2, P = 0.223). The same holds true when only the phylogenetically informative characters for the ingroup comparison are tested ($\chi^2 = 3.38$, df = 2, P = 0.184), although it approaches significance when the outgroup is added ($\chi^2 = 4.94$, df = 2, P = 0.084). Overall, while adding the outgroup taxa increases the absolute numbers of variable

Table 2. Percent of variable and phylogenetically informative sites of cytochrome oxidase subunits I, II, and III^a

	COI		COII		COIII		Combined COI, II, III	
	Ingroup	Including outgroup	Ingroup	Including outgroup	Ingroup	Including outgroup	Ingroup	Including outgroup
Total positions	408		688		419		1,515	
Variable	23.3 (95)	28.2 (115)	20.5 (141)	24.4 (168)	25.1 (105)	28.6 (120)	22.5 (341)	26.6 (403)
1st	3.2 (13)	4.9 (20)	2.9 (20)	4.1 (28)	4.1 (17)	5.0 (21)	3.3 (50)	4.6 (69)
2nd	0.2 (1)	0.2 (1)	0.6 (4)	1.0 (7)	0.7 (3)	0.9 (4)	0.5 (8)	0.8 (12)
3rd	19.9 (81)	23.1 (94)	17.0 (117)	19.3 (133)	20.3 (85)	22.7 (95)	18.7 (283)	21.2 (322)
Phylogenetic	15.0 (61)	21.3 (87)	12.2 (84)	16.6 (114)	14.3 (60)	19.6 (82)	13.5 (205)	18.7 (283)
1st	1.2 (5)	3.9 (16)	1.2 (8)	2.8 (19)	2.4 (10)	3.3 (14)	1.5 (23)	3.3 (49)
2nd	0 (0)	0 (0)	0.1 (1)	0.4 (3)	0.2 (1)	0.5 (2)	0.1 (2)	0.3 (5)
3rd	13.8 (56)	17.4 (71)	10.9 (75)	13.4 (92)	11.7 (49)	15.8 (66)	11.9 (180)	15.1 (229)

^a Values in parentheses indicate the number of positions. The ingroup taxa are represented by 12 haplotypes while the outgroup comparison also includes the *D. yakuba* and *D. melanogaster* sequences

and phylogenetically informative sites, it does not affect the relative number of characters among the subunits. As can be seen from Table 2, most of the positions that have changed are 3rd position sites, which is expected since substitutions in most 1st and 2nd codon positions result in nonsynonymous changes.

Table 3 shows base composition and base composition bias. The base composition bias statistic is calculated according to Irwin et al. (1991) and ranges in value from between 0 to 1; zero indicating no bias, and one showing complete base composition bias. As has been reported for many insect mtDNA protein-coding genes (Clary and Wolstenhome 1985; DeSalle et al. 1987; Simon et al. 1994), the 1st and 2nd codon positions have a much higher GC content than do the 3rd positions. It appears that there are differences among the three subunits in regards to base compositional bias (however, these are not statistically evaluated due to the confounding effects of phylogeny). When considering all the sites, COI has the least amount of bias, followed by an intermediate value for COII, and finally COIII having the greatest compositional bias. However, for phylogenetic reconstruction it is the variable and phylogenetically informative positions that are important. Table 3 reveals a noticeable difference in the amount of bias between all positions versus variable or phylogenetically informative sites. Accordingly, this needs to be taken into account when base composition is used in genetic distance or maximum likelihood estimation. Nonetheless, the relative ordering among the cytochrome oxidase subunits based on the level of compositional bias remains the same as when considering all the positions.

Intraspecific and interspecific variation among strains and species depended on the comparison. Three species were examined for intraspecific variation, with two strains surveyed for each. The strains of *D. borborema* showed no variation; however, they also came from the same locality. Both the *D. martensis* and *D. serido* strains were collected from different localities and

showed some variation, with the *D. martensis* haplotypes differing by 2.6% and *D. serido* by 6.6%. The large sequence divergence between *D. serido* haplotypes may be indicative of the fact that this taxon appears to represent a superspecies (Tido-Sklorz and Sene 1995). Comparisons within species clusters range from 1.7–9.6% divergence, with variation among species clusters ranging from 8.6–10.1%. Sequence divergence levels between the *buzzatii* species complex and the outgroup taxa range from 11.8–13.9%.

The branch-and-bound unweighted parsimony analysis resulted in one most parsimonious tree, which is presented in Fig. 2. This tree has a length of 755, a consistency index (CI) of 0.638 and a rescaled consistency index (RCI) of 0.356. The bootstrap values for the unweighted analysis indicate relatively strong support at all nodes, using the 70% or greater criterion of Hillis and Bull (1993), except for those defining the *stalker* cluster. When transversion parsimony is used (Brown et al. 1982; Swofford and Olsen 1990), three most parsimonious trees are produced with lengths of 342, which are presented in Fig. 3A–C. One tree is the same as the unweighted parsimony tree, and all three trees differ only in the arrangement of the *stalker* cluster. Once again, bootstrap support at most nodes is relatively strong except for the *stalker* cluster, and in this instance, the placement of *D. martensis* in the *martensis* cluster.

Distance estimates produce different trees depending on which distance procedure is used. The standard Kimura 2-parameter (Kimura 1980) estimates when clustered by using a heuristic version of the minimum evolution method produce the tree in Fig. 4, which is different from any of the parsimony based trees. This tree is generated either by using all substitutions or by just using transversional differences. One problem with the analysis of mtDNA is the problem of base compositional bias, which can lead to the underestimation of the true amount of divergence (Saccone et al. 1989, 1993; Collins et al. 1994). Two distance procedures have been devel-

Table 3. Percentage base composition and bias for cytochrome oxidase subunits I, II, and III^a

	1st	2nd	3rd	All	Var	Phy
Cytochrome oxidase I						
A	0.233	0.133	0.446	0.271	0.353	0.307
C	0.174	0.301	0.084	0.186	0.157	0.188
G	0.301	0.162	0.024	0.162	0.037	0.027
T	0.291	0.404	0.445	0.380	0.453	0.477
Bias	0.123	0.273	0.522	0.202	0.408	0.379
Cytochrome oxidase II						
A	0.303	0.270	0.422	0.331	0.292	0.291
C	0.172	0.186	0.061	0.140	0.137	0.163
G	0.228	0.135	0.018	0.127	0.038	0.033
T	0.296	0.410	0.498	0.401	0.532	0.513
Bias	0.132	0.239	0.560	0.310	0.432	0.405
Cytochrome oxidase III						
A	0.271	0.203	0.425	0.300	0.322	0.325
C	0.170	0.261	0.067	0.166	0.115	0.143
G	0.221	0.137	0.014	0.124	0.050	0.042
T	0.338	0.399	0.492	0.409	0.513	0.490
Bias	0.145	0.213	0.558	0.279	0.466	0.420

^a Values are calculated according to coding position (1st, 2nd, 3rd), all positions (All), only variable positions (Var), and only phylogenetically informative positions (Phy). The bias is calculated using the formula of Irwin et al. (1991)

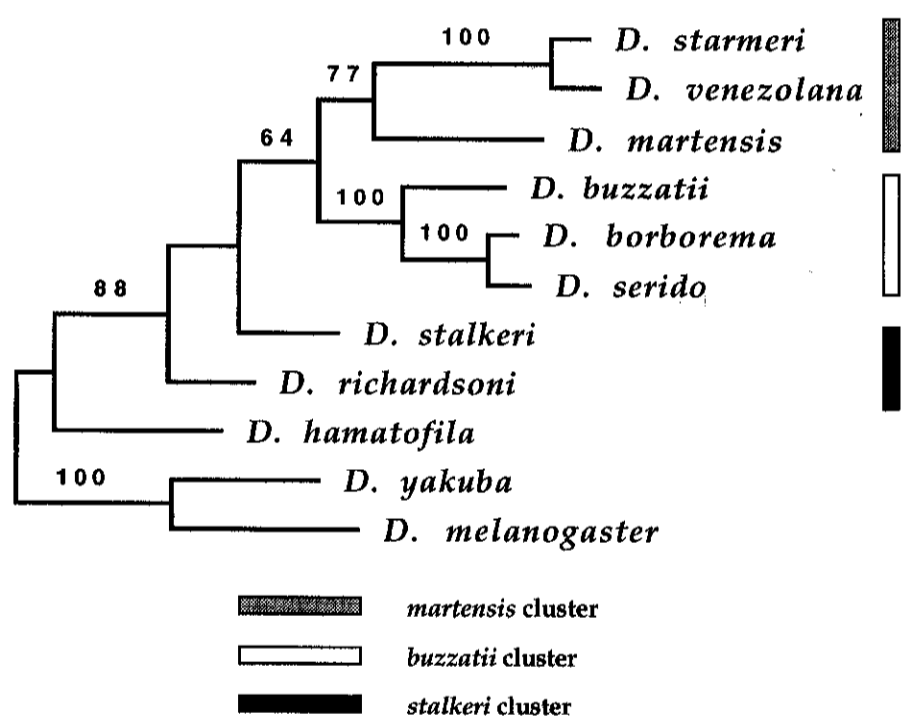


Fig. 2. Most parsimonious tree produced by the branch-and-bound algorithm using all the cytochrome oxidase sequence. The tree length is 755 with a consistency index of 0.638 and a rescaled consistency index of 0.356. Values at the nodes indicate percentage bootstrap support (only values greater than 50% are presented).

oped that potentially can correct compositional bias problems, and both of these were performed. One procedure, the Tamura–Nei distance (Hasegawa et al. 1985; Tamura and Nei 1993), was developed to compensate for overall base composition bias presuming that all taxa have approximately the same nucleotide composition. The other distance measure, the Log Det/Paralinear procedure (Lockhart et al. 1994; Lake 1994), was designed to adjust for base composition bias when the nucleotide bias differs among taxa. The Tamura–Nei distance gives the same tree as the Kimura distance in Fig. 4, both when

using all substitutions and when using only transversional changes. The Log Det/Paralinear procedure also produces the same tree as in Fig. 4. The percentage bootstrap support values for the distance analyses discussed above are presented in Table 5.

Another potential difficulty with distance estimation is whether the data meet the assumptions of the model. In this instance, these data do not appear to meet the Poisson expectation, which is the distribution underlying most of the commonly used distance estimation procedures. This can be shown by testing whether the distribution of character changes fits a Poisson distribution, or is more appropriate under a Γ -distributed rates model. To test for this, the most parsimonious tree (Fig. 2) was evaluated by using the chart option of MacClade (Maddison and Maddison 1992), which calculates the number of character changes over the tree based on the most parsimonious reconstruction. The resulting distribution was evaluated by using the maximum-likelihood program GAMMA (Sullivan et al. 1995), which tests whether the distribution fits a Poisson or Γ -distributed rates model, and in addition, calculates the shape parameter α for the Γ -distribution. In this instance, the tree in Fig. 2 rejects a Poisson distribution ($\chi^2 = 356$, $df = 1$, $P < 0.0001$), and estimates the shape parameter α to be 0.384. One way to get around the distribution problem is to use only positions that potentially do meet the assumptions of the model, which in this instance would be calculating the distance based on only 3rd codon positions. When this is done with the Kimura 2-parameter distance, the tree in Fig. 6 is produced, which is the same as the transversion parsimony tree of Figure 3C. Using only the

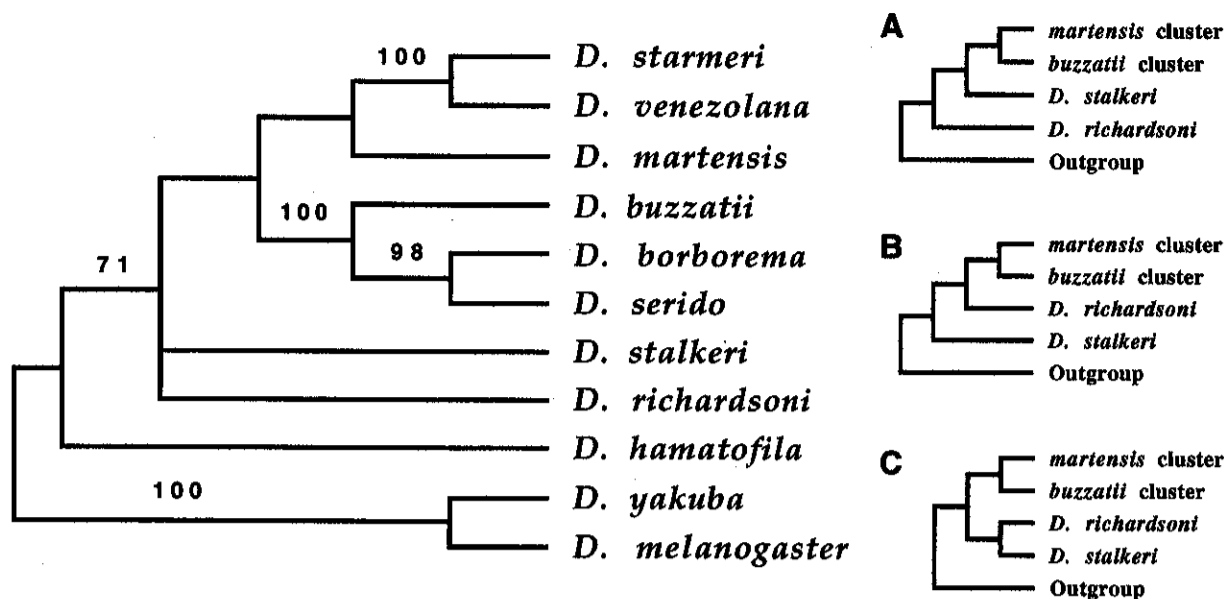


Fig. 3. Strict consensus tree of the three most parsimonious trees inferred by transversion parsimony using all the cytochrome oxidase sequence. The length of these trees is 342. Values at the nodes indicate percentage bootstrap support (only values greater than 50% are presented). Panels A–C show the three trees, which differ only in the arrangement of the *stalker* cluster.

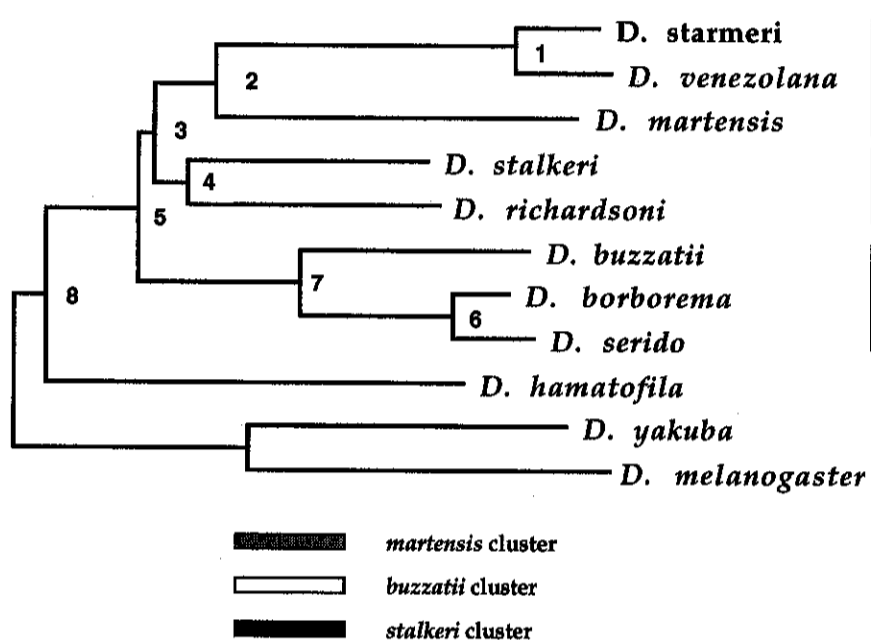


Fig. 4. Kimura 2-parameter distance tree produced by a heuristic search using the minimum evolution method with all the cytochrome oxidase sequence. The same branching pattern is also obtained with the minimum evolution method when the Kimura 2-parameter distance is applied to only 3rd positions (only transversion substitutions), the Tamura–Nei distance (all substitutions and only transversions), and the Log Det/Paralinear distance are utilized. Numbers at the nodes correspond to the numbers in Table 5, which give the percentage bootstrap support of each of the different analyses.

3rd codon positions with the Tamura–Nei distance and the Log Det/Paralinear procedure is not possible since some of the distances produced were undefined. When the phylogeny is estimated by using the Kimura 2-parameter distance modified for the Γ -distribution (Jin and Nei 1990), the answer depends on the value of the shape parameter that is employed. If the shape parameter α of 0.384 is used, which is the estimate of the parsimony procedure from above, the tree that is produced is the same as the Poisson-distributed Kimura distance tree in Fig. 4. But when an α of 0.117 is used, which is the value for the shape parameter estimated by the maximum-likelihood method from below, then the branching pattern of Fig. 3B is produced.

The maximum-likelihood estimate for the four trees was performed by using the 2-parameter model for un-

equal base frequencies of Hasegawa et al. (1995), with a discrete approximation to the Γ -distribution, and with the transition/transversion ratio and the shape parameter estimated according to the model. A total of 10 rate categories was used (Yang 1994), and the average rate for each category was represented by the mean. Since the total base composition does not reflect the base composition of the positions that are free to vary (Table 3), the base composition used in the maximum-likelihood calculations was based only on the base frequencies of the variable positions (A = 0.318, C = 0.136, G = 0.041, T = 0.505). The likelihood scores and parameter estimates are given in Table 4.

Finally, the Templeton parsimony test (Templeton 1983; Kishino and Hasegawa 1989) was performed on the four phylogenetic hypotheses inferred from all the different analyses (Figs. 2–5). This test is conducted by calculating a mean and variance of step differences among trees, so that if the mean difference in steps between a tree and the best tree is greater than 1.96 standard deviations, then the trees are considered statistically different. The results of this test reveal that there is no statistical difference among the four trees (Table 4). However, this is a conservative test which calculates a variance based only on the unweighted characters, so transitions and transversional changes are considered equally.

Examining each of the subunits in separate phylogenetic analyses reveal some interesting patterns (Fig. 6). They show that in both the unweighted and transversion parsimony analyses, COIII appears to produce the best result, followed by COII and then by COI, which produces the worst, relative to what can be considered the best estimate of the phylogeny (the three parsimony trees from above). When the COI subunit is analyzed using unweighted parsimony, four trees are produced, but none of these four trees is congruent with any of the trees considered as the best estimate of the phylogeny. The unweighted analysis produced a RCI = 0.332 which is

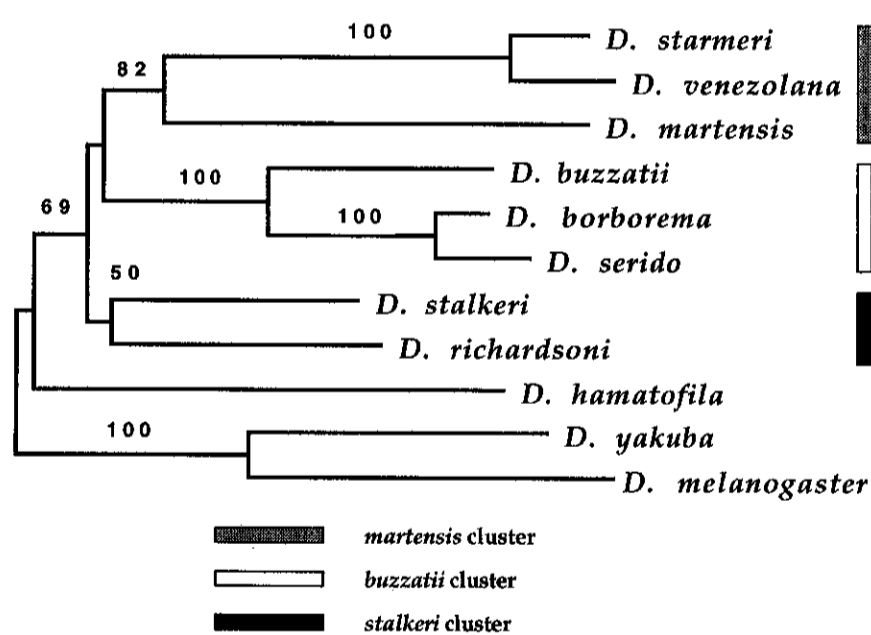
Table 4. The maximum-likelihood values, parameter estimates, and results of the Templeton parsimony test for each of four phylogenetic hypotheses inferred from all different analyses

Tree	Likelihood score	Ts/Tv ratio	α shape parameter	Steps	Difference in steps	SD	Worse?
Fig. 2, 3A	-5595.16880	7.22	0.113	755	Best		
Fig. 3B	-5595.16880	7.22	0.113	759	4	2.829	No (P = 0.157)
Fig. 3C, 5	-5595.16767	7.21	0.113	757	2	3.163	No (P = 0.527)
Fig. 4	-5560.09203	7.82	0.111	760	5	4.360	No (P = 0.251)

Table 5. The bootstrap percentage values for Fig. 4^a

Node	Kimura 2-parameter			Tamura-Nei		Log Det/ Paralinear All
	All	Tv only	3rd only All	All	Tv only	
1	100	100	100	100	100	100
2.	81	65	61	74	70	53
3.	—	—	—	—	—	—
4.	—	70	71	60	80	—
5.	85	89	78	95	92	96
6.	100	100	100	100	100	100
7.	100	10	100	100	100	100
8.	100	100	100	100	100	100

^a Node refers to the numbered nodes presented in Fig. 4. Bootstrap percentage values of less than 50% are represented by a dash. All indicates that both transition and transversion substitutions were used in the analysis; T_v indicates that only transversion substitutions were included. See text for details of analyses

**Fig. 5.** Kimura 2-parameter distance tree produced by a heuristic search using the minimum evolution method with all the cytochrome oxidase sequence and when considering only 3rd positions (all substitutions). Values at the nodes indicate percentage bootstrap support (only values greater than 50% are presented).

the lowest value for any of the analyses performed, and in addition, the bootstrap support for most nodes is not strong. The results are the same when considering the transversion parsimony analysis. Three equally parsimonious trees are generated and none of these trees is congruent with best estimate trees. Once again, bootstrap support is very poor except for a few nodes. The results for COII appear intermediate in that both the unweighted and transversion parsimony analyses produce a single

most parsimonious tree and in both instances these trees are congruent with what is considered the best estimate of the phylogeny. The unweighted parsimony analysis produced a RCI = 0.384 and the bootstrap values are better than for the COI result, although the bootstrap support for the transversion parsimony tree is slightly worse than the COI case. The COIII subunit indeed appears to produce the best results. Both the unweighted and transversion parsimony analyses produced one most parsimonious tree that is congruent with the best estimate of the phylogeny. The unweighted tree has a RCI = 0.386 which is the greatest for any of the analyses. Bootstrap support is strong for both the unweighted and transversion parsimony analyses. When the subunits are combined for analysis the results seem to show an additive effect relative to the separate analyses (not shown). In both instances, when COI is combined with either COII or COIII, the RCI and bootstrap values are intermediate to values obtained when analyzing the respective subunits separately. The COI and COII combination produce one most parsimonious tree for the unweighted analysis and three trees for the transversion parsimony analysis; none of these are congruent with the best phylogeny estimate. The RCI is 0.350 with the bootstrap support only slightly better than in the separate COI analysis. The COI and COIII combination produces about the same results. The unweighted analysis infers one tree (like Fig. 3A) which is congruent with the best estimate of the phylogeny, but the single most parsimonious tree in-

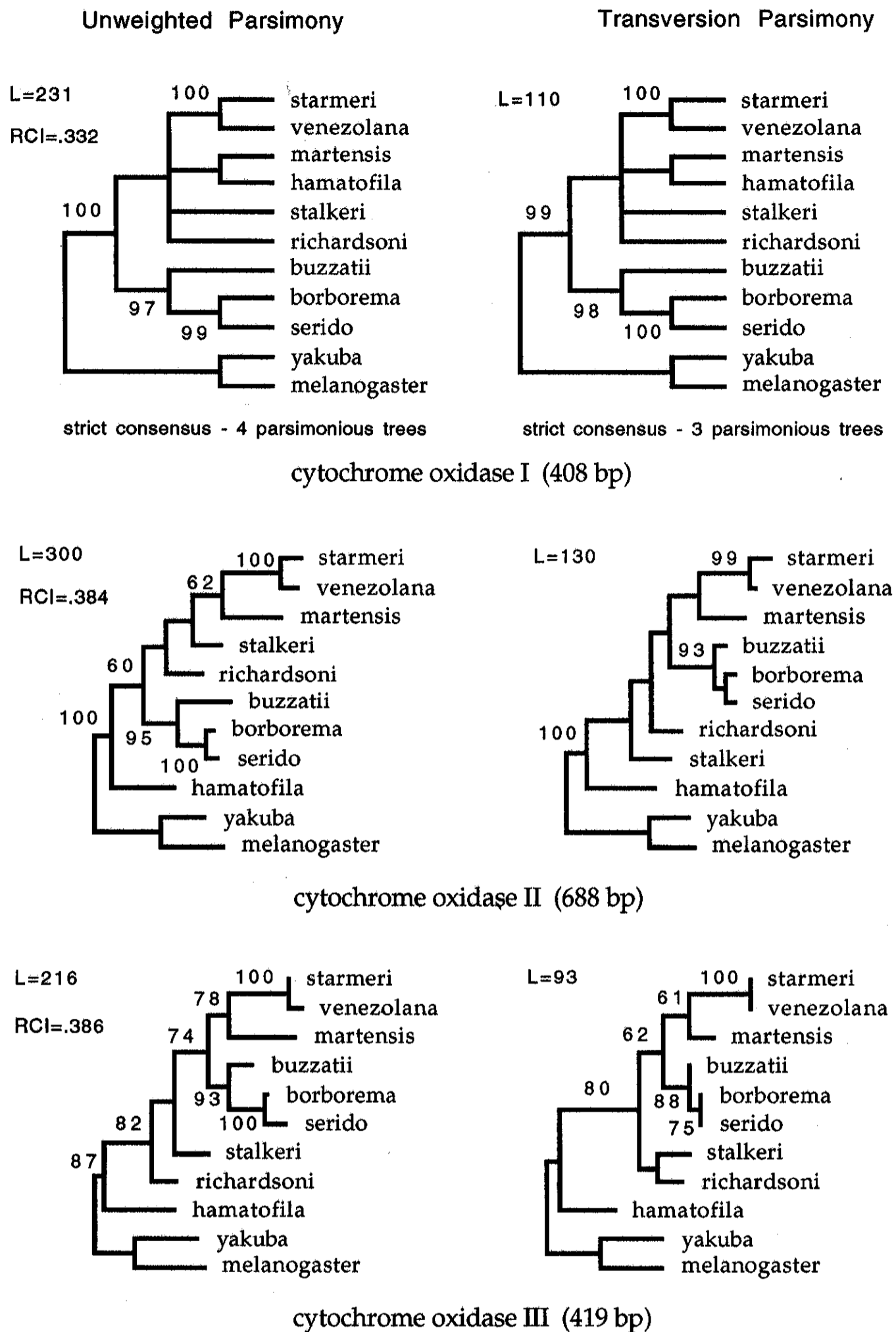


Fig. 6. Unweighted and transversional parsimony trees for each of the cytochrome oxidase subunits analyzed separately. Values at the nodes indicate percentage bootstrap support (only values greater than 50% are presented). The most parsimonious tree is presented, except

when multiply parsimonious trees were produced, in which case the strict consensus tree for the equally parsimonious trees is shown. The tree length (*L*) and rescaled consistency index (*RCI*) is presented for each tree.

ferred by transversion parsimony is not congruent. The *RCI* = 0.341 is even lower than the COI and COII combination, and once again bootstrap support is weak. As expected from the separate analyses, the best combination appears to be COII with COIII. The unweighted analysis produces one parsimonious tree (like Fig. 3A) and the transversion parsimony analysis produces two most parsimonious trees (like Fig. 3B and C), all of

which are congruent with the best phylogeny estimate. The *RCI* is 0.383, which is intermediate between the separate COII and COIII analyses. However, the bootstrap support is the greatest for any of the analyses, including the case when all subunits are considered.

In an effort to determine the possible underlying cause for this difference among subunits, each subunit was analyzed separately to determine whether the distribution

Table 6. The α values for all changes and for only transversion substitutions as determined by parsimony method^a

	COI		COII		COIII	
	All	Tv only	All	Tv only	All	Tv only
Entire sequence	0.346*	0.171–0.216*	0.371*	0.137–0.161*	0.481*	0.179–0.184*
1st	0.343*	10 ⁶	0.387*	10 ⁶	0.242*	10 ⁶
2nd	10 ⁵	10 ⁵	0.067*	10 ⁵	0.029*	0.005*
1st and 2nd	0.152*	10 ⁶	0.198*	10 ⁶	0.121*	0.028*
3rd	3.448*	0.869–1.414*	3.362	0.632–0.832*	27.4	1.130–1.224*

^a Values with an asterisk indicate that they are significantly different from a Poisson distribution as determined by a likelihood-ratio test. Very large values of α are expressed only to the correct order of magnitude. When more than one parsimonious reconstruction of changes is possible on the tree, two values of α are presented, which represent the two most extreme reconstructions

of nucleotide substitutions fits a Poisson or Γ -distributed rates model. This was accomplished by the same method as described above, using the most parsimonious tree (Fig. 2) and generating the number of character changes over the tree with MacClade (Maddison and Maddison 1992). Once again, the resulting distributions were evaluated by using the maximum-likelihood program GAMMA (Sullivan et al. 1995). The results of these analyses are presented in Table 6.

Discussion

The molecular evidence presented here suggests that three trees should be considered as the best estimate of the phylogeny of the *D. buzzatii* complex, and currently we cannot distinguish which one is best. These three trees result from the parsimony analyses and some of the distance analyses, and they are the trees with the highest maximum-likelihood scores as well (Table 4). Importantly, they are fully congruent with the chromosomal inversion phylogeny of Ruiz and Wasserman (1993). This confirms previous molecular studies which have revealed a high degree of congruence between molecular phylogenies and chromosomal inversion phylogenies (MacIntyre and Collier 1986; Spicer 1992). The congruence appears to represent a justification for using mitochondrial cytochrome oxidase for reconstructing lower level phylogenies, and reinforces the results of Beckenbach et al. (1993) showing that the cytochrome oxidase subunits can be useful for reconstructing lower level phylogenies of *Drosophila*, and probably other insects. Interestingly, a fourth tree that is not congruent with the chromosomal inversion phylogeny is estimated by some of the distance procedures. However, when the proper distributional assumptions are met, the distance techniques then produce one of the trees that are inferred by parsimony and are congruent with the chromosomal phylogeny. This shows the importance of meeting the assumptions of the distance procedures before they are used for phylogenetic inference.

The separate analyses of the subunits reveal some

interesting observations regarding the evolution and phylogenetic utility of cytochrome oxidase. These analyses suggest that not all the subunits have the same phylogenetic information content. This observation has potential implications for insect systematics, since the cytochrome oxidase subunits are increasingly being used for phylogenetic reconstruction (e.g., Brower 1994; Sperling and Hickey 1994; Brown et al. 1994). The result that the subunits appear to contain differing amounts of phylogenetic information depends partly on the assumption that we have inferred the correct phylogeny. The justification that we have indeed inferred the correct phylogeny is partly based on congruence. Given that independent data sets (molecular, morphological, and chromosomal) infer the same phylogeny supports the conclusion that the correct phylogeny has been determined. If the three trees from above can be taken as a likely estimate of the phylogeny, then these trees can be used for comparison against the separate analyses of the cytochrome oxidase subunits. When this is done several interesting observations are apparent. Foremost, not all the subunits seem to contain the same phylogenetic information, since COIII resolves better with higher bootstrap support than either COI or COII (at least for the sections of COI and COIII sequenced for this study; COII was entirely sequenced). This is contrary to what might be anticipated based on the summary statistics presented for each subunit (Tables 2 and 3). The most obvious explanation would be that the sequence with the greatest length should provide the best estimate, particularly since it was shown that all the subunits appear to contain the same relative amount of variable positions (Table 2). However, COIII (419 bp) is far from the largest fragment sequenced, with COII (688 bp) being much longer, and COI (408) about comparable in length. Along the same lines, it is usually assumed that a better phylogenetic answer will be obtained with additional sequences, unless the added sequences are known to be inappropriate for the evolutionary problem in question (substitution rate too fast or too slow). However, in this instance it seems that by adding what appears to be appropriate sequence (COI) may actually be adding misinformation. When the separate subunits are added to-

gether for the combination analyses the results from these combinational data sets are very consistent. Whenever COI is included as part of the data set both the resolving ability and resultant bootstrap support for the nodes are greatly diminished. This is true even for the entire data set. As the results indicate, the COII and COIII combination data set give a much more strongly supported inference than does the combination of all subunits COI-III. Another anomaly is that of base composition bias. It has been known for some time that when the base frequency is unequal then phylogenetic reconstruction can be affected (Saccone et al. 1989). Insect mitochondrial DNA has long been known to have a strong base composition bias (Clary and Wolstenhome 1985; DeSalle et al. 1987), and as expected the cytochrome oxidase genes examined here show this pattern. However, base composition bias cannot explain the apparent superiority of COIII either. In this instance, COIII appears to have the most biased base composition for both the variable and phylogenetically informative positions (Table 3), at least according to the base composition bias statistic that has been employed in this study, so this does not seem to be an adequate explanation.

An explanation for these results can potentially be offered that relates to rate heterogeneity. It has been shown that extreme nucleotide substitution rate heterogeneity can present difficulties for all methods of phylogeny reconstruction (Sullivan et al. 1995). The analyses presented in Table 6 suggest that the estimated distribution of rates is different among the three subunits. As would be predicted by the phylogenetic reconstruction results presented in Fig. 6, COI and COII have the highest degree of rate heterogeneity and COIII has the least. This is true for the entire sequence when all changes are considered (although not for transversal substitutions), but especially when only 3rd codon positions are examined. In fact, it appears that 3rd positional changes in COIII are better explained by a Poisson distribution, while the 3rd codon positions in COI and COII fit a Γ -distributed rates model. The importance of the 3rd codon position is that most of the phylogenetic information in these subunits comes from 3rd positional changes (Table 2), because 1st and 2nd codon position changes usually result in amino acid substitutions that are highly constrained in these genes. The reason for this rate heterogeneity may be due to a difference in the rate of nucleotide substitution at different amino acids. The subunits do have different amino acid compositions, and it does appear that nucleotide substitutions at some amino acids are phylogenetically more informative than at others. In particular, it seems that amino acids with 4-fold degenerate sites are better for phylogenetic reconstruction than are amino acids that have 2-fold degenerate sites. Still, this is not an absolute rule since it appears that nucleotide substitutions at 3rd codon positions in histidine are the most phylogenetically informative in this

data set (as determined by having the highest rescaled consistency index value; results not shown), but this amino acid is in the 2-fold degenerate class. At the moment, it cannot be discerned if the underlying cause for the molecular evolutionary differences among the subunits is due to either amino acid composition differences among the subunits, or to different mutation or selection regimes on the subunits themselves. Nevertheless, it does appear that the observed difference in phylogenetic resolving ability among the subunits can be explained by the corresponding differences in rate heterogeneity.

These results appear to contradict some of the usual caveats expressed when analyzing DNA sequence data sets. It seems that none of the most commonly discussed difficulties with molecular data sets (amount of sequence variation, sequence length, and base composition bias) offer an adequate understanding of the difference in phylogenetic resolution among the cytochrome oxidase subunits. However, the degree of rate heterogeneity among the subunits does seem to offer some explanation. The underlying reasons for this heterogeneity have not yet been elucidated. Nevertheless, it appears that rate heterogeneity is an important factor that needs to be taken into consideration when using molecular sequences for phylogeny reconstruction. Notwithstanding these difficulties, it does appear that mitochondrial protein-coding genes can reconstruct relationships among relatively closely related taxa, although it remains to be shown over what range of divergence these genes can adequately infer insect phylogenies. Consequently, further study will be necessary to determine the importance of the different parameters involved.

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