

Molecular Phylogeny of the *Drosophila virilis* Species Group (Diptera: Drosophilidae) Inferred from Mitochondrial 12S and 16S Ribosomal RNA Genes

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ABSTRACT The phylogenetic relationships among all members of the *Drosophila virilis* Sturtevant species group were inferred from mitochondrial DNA [partial 16S ribosomal RNA (rRNA), transfer RNA (tRNA)^{val}, and partial 12S rRNA] sequence data. These data show support for four lineages within the *D. virilis* species group, which are consistent with previous phylogenetic work performed on the group. However, the relationship among these four groups is not well resolved. These data also suggest that the species *D. borealis* Patterson is not a monophyletic assemblage, and that the disjunct distribution actually represents two genetically distinct species.

KEY WORDS *Drosophila virilis* species group, maximum likelihood, molecular clock, mtDNA

OVER THE PAST 50 yr, the *Drosophila virilis* Sturtevant species group has been regarded as one of the best model systems for the study of speciation (Patterson and Stone 1952, Mayr 1963, Wiley 1981, Coyne and Orr 1989), molecular evolution (Nei 1971, Cohen and Bowman 1979, Throckmorton 1982), and the evolution of mating behavior (Spieth 1951, Patterson and Stone 1952, Watanabe and Kawanishi 1979). Within the group there are fourteen species currently recognized, which have been further divided into two clades or “phylads”: the *D. virilis* phylad and the *D. montana* Stone, Griffen & Patterson phylad (Throckmorton 1982, Spicer 1991). Recently, the *D. virilis* group has been further separated into four lineages (Spicer 1992); the *D. virilis* phylad, the *D. montana* subphylad, the *D. littoralis* Meigen subphylad, and the *D. kanekoi* Watabe & Higuschi subphylad (Table 1).

In this article we present a molecular phylogeny, using a complete sample of species of the *D. virilis* group, from mitochondrial DNA sequence data encompassing the partial 3' region of the 12S ribosomal RNA (rRNA) gene, the complete transfer RNA (tRNA)^{val}, and the 5' region of the 16S rRNA. This region has been shown to be phylogenetically informative in inferring relationships among closely related species of Hawaiian crickets in the genus *Laupala* (Shaw 1996). For the first time, molecular sequence data from a recently described species *D. canadiana* Takada & Yoon are also presented here.

Materials and Methods

Samples. Representative species of the entire *D. virilis* group were sampled for phylogenetic analysis (Table 1). The species *D. arizonae* Ruiz, Heed & Wasserman was used as an outgroup for this study. In addition, published sequences from *D. yakuba* Burla (Clary and Wolstenholme 1985; Genbank NC001322) and *D. melanogaster* Meigen (Lewis et al. 1995; Genbank NC001709) were used for comparative purposes.

DNA Extraction and Amplification. Total genomic DNA was extracted from live flies by using the phenol/chloroform method described by Spicer (1995). Portions of both the mtDNA 16S and 12S regions of rDNA as well as an intervening region that codes for the transfer RNA (tRNA)^{val} were sequenced for all members of the *D. virilis* species group. Amplification primers for this region were designed to correspond to that of Shaw (1996). Primers correspond to *D. yakuba* mtDNA (Clary and Wolstenholme 1985) sites 14588–14608 (12Sai, 5'-AAA CTA GGA TTA GAT ACC CTA TTA T) and site 13309–13331 (16Ssb, 5'-CTA ATG ATT ATG CTA CCT TTG CAC A). Double stranded copies of all regions were amplified using the polymerase chain reaction (PCR) in 50- μ l volume reactions. All reactions were heated at 94°C for three minutes. The reactions were then subjected to 34 cycles in a PE 480 thermocycler (Perkin-Elmer, Norwalk, CT). Each cycle consisted of 1.5 min at 94°C denaturation, 2 min at 48°C annealing, and 3 min at 72°C extension. Amplified PCR products were cleaned before sequencing using a polyethylene glycol precipitation.

Sequencing. All sequencing was done using dye terminator cycle sequencing following the protocol

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Table 1. Taxonomic samples used in this study

| Taxonomic grouping | Species | Strain | Collection locality | Genbank acc. no. |
|-------------------------------------|--------------------------------------|---------------|--|------------------|
| virilis phylad | <i>Drosophila americana</i> | 15010-0951.14 | USA: Independence, Missouri (Throckmorton) | AF185054 |
| | <i>Drosophila texana</i> | 15010-1041.26 | USA: Tallahassee, Florida | AF185057 |
| | <i>Drosophila novamexicana</i> | 15010-1031.7 | USA: Patagonia, Arizona | AF185055 |
| | <i>Drosophila lummei</i> | 15010-1011.2 | SWEDEN: Overhalix | AF185056 |
| | <i>Drosophila virilis</i> | — | JAPAN: Sapporo | AF185058 |
| montana phylad montana subphylad | <i>Drosophila borealis</i> (eastern) | 15010-0961.2 | USA: Minnesota, Itasca Park | AF185059 |
| | <i>Drosophila borealis</i> (western) | 15010-0961.3 | USA: Idaho: Chester | AF185062 |
| | <i>Drosophila flavomontana</i> | 15010-0981.3 | USA: Colorado: Hamilton | AF185061 |
| | <i>Drosophila lacicola</i> | 15010-0991.13 | Canada: Manitoba | AF185060 |
| | <i>Drosophila montana</i> | 15010-1021.14 | USA: Utah: Cottonwood Canyon | AF185063 |
| ezoana subphylad | <i>Drosophila ezoana</i> | 15010-0971.0 | Japan: Hokkaido Raus | AF185065 |
| | <i>Drosophila littoralis</i> | 15010-1001.1 | Switzerland: Vitznau | AF185066 |
| | <i>Drosophila canadiana</i> | 15010-1091.0 | Canada: British Columbia: Smithers | AF185067 |
| kanekoi subphylad | <i>Drosophila kanekoi</i> | 15010-1061.0 | Japan: Sapporo | AF185064 |
| repleta species group—outgroup | <i>Drosophila arizonae</i> | 15081-1271.7 | Mexico: Hidalgo | AF185068 |

Strain numbers correspond to the National *Drosophila* Species Resource Center stock numbers, where applicable.

specified by the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Revision B, August 1995, Perkin-Elmer). Primers used for amplification served as sequencing primers. Additional internal primers were designed for sequencing purposes (12Sbi, 5'-AAG AGC GAC GGG CGA TGT GT and 12Sbr-S, 5'-ACC GCC CGT CAC CCT C).

Sequence Alignment. Consensus sequences were built from sequence fragments using the computer package Sequencher (Gene Codes Corporation 1994). All sequences were aligned visually. Sequences have been deposited into GenBank (Table 1 for accession numbers), and the aligned data matrix is available upon request from the authors.

Preliminary Sequence Analysis. Sequences were evaluated for overall base composition bias and among taxon base composition. The base composition bias statistic was calculated according to Irwin et al. (1991) and ranges in value from 0 to 1; zero indicating no bias and one showing complete base composition bias. These results are important when choosing an appropriate maximum likelihood and distance model because an extreme overabundance of one nucleotide state can increase the tendency for those sites to become saturated (Irwin et al. 1991). In addition, a

strongly skewed mutation bias can violate the assumptions of parsimony (Perna and Kocher 1995, Spicer 1995, Yoder et al. 1996).

Phylogenetic Analysis. Maximum parsimony searches were conducted using the branch-and-bound algorithm, which guarantees the shortest tree. To assess confidence in the resulting tree topologies, bootstrap tests (Felsenstein 1985, Hillis and Bull 1993) were performed using 300 replicates.

In addition to searching for trees under the maximum parsimony criterion, we also searched for trees using maximum likelihood and distance (minimum evolution criterion). To determine which model best fit the data, a series of nested [i.e., the null hypothesis (H0) is a special case of the alternative hypothesis (H1)] hypotheses were performed on various nucleotide substitution models (see Table 2). A likelihood ratio test (LRT) was then performed (Goldman 1993). We calculated the test statistic as $2(\ln L_0 - \ln L_1) = -2 \ln 76$, where L0 and L1 are the likelihood values under the null and alternative hypotheses, respectively. We calculated the associated probability using a chi-square distribution with the degrees of freedom equal to the difference in number of free parameters between the two models. We found that a HKY-Γ

Table 2. Maximum likelihood analysis of hierarchical substitution models for the distance tree (Fig. 2)

| H ₀ vs. H ₁ | -LnL ₀ | -LnL ₁ | -2lnΛ | df | P |
|-----------------------------------|-------------------|-------------------|--------|----|----------|
| JC vs. F81 | 3105.45015 | 2824.75509 | 561.39 | 3 | <0.0001* |
| F81 vs. HKY85 | 2824.75509 | 2786.81025 | 83.88 | 1 | <0.0001* |
| HKY85 vs. HKY85+Γ | 2786.81025 | 2694.59904 | 184.42 | 1 | <0.0001* |
| HKY85+Γ vs. GTR+Γ | 2694.59904 | 2693.85688 | 1.48 | 1 | 0.2238 |
| HKY85+Γ vs. HKY85+I+Γ | 2694.59904 | 2694.59904 | 0 | 1 | 1.000 |
| HKY85+Γ (clock enforced) | 2694.59904 | 2702.92939 | 16.66 | 13 | 0.2153 |

* Hypothesis rejected.

Likelihoods were evaluated with the likelihood ratio test (LRT) as described in the Methods and Materials section. The abbreviations for the models and parameters are: JC, Jukes and Cantor (1969); F81, Felsenstein (1981); HKY, Hasegawa, Kishino, and Yano (1985); GTR, general time-reversible model (Lanave, et al., 1984; Tavaré, 1986; Rodriguez et al., 1990); Γ, shape parameter of the gamma distribution estimated with 10 rate categories; I, proportion of invariable sites. Degrees of freedom when testing the hypothesis of a molecular clock equal n-2, where n = the number of taxa sampled (Felsenstein, 1993).

Table 3. Base composition bias statistic calculated according to Irwin et al. (1991)

| | 16S rRNA | (tRNA) ^{VAL} | 12S rRNA | All positions | Variable positions |
|-------------------|----------|-----------------------|----------|---------------|--------------------|
| No. of aligned bp | 783 | 73 | 455 | 1,311 | 136 |
| A | 0.428 | 0.395 | 0.365 | 0.404 | 0.343 |
| C | 0.096 | 0.118 | 0.156 | 0.117 | 0.099 |
| G | 0.046 | 0.086 | 0.103 | 0.068 | 0.043 |
| T | 0.430 | 0.400 | 0.103 | 0.409 | 0.513 |
| Bias | 0.477 | 0.395 | 0.321 | 0.419 | 0.536 |

model best fit our data (Table 2). Next, we performed a heuristic search, under the maximum likelihood and minimum evolution optimality criteria, using the same branch swapping techniques as described when using maximum parsimony. The α -shape parameter of the gamma distribution was estimated using maximum likelihood. Bootstrap tests were once again performed using 300 replicates. All maximum parsimony, minimum evolution and maximum likelihood analyses were performed using PAUP*beta4.0a (Swofford 2000).

To test the null hypothesis of a molecular clock for our data set, an additional LTR was performed. Once again, the likelihood test statistic is assumed to be approximately equal to a chi-square distribution with $n-2$ degrees of freedom (where n equals the number of taxa sampled, Felsenstein 1981).

Results

Simple Sequence Statistics. An aligned 1311 bp fragment was sequenced for a region spanning the 5' part of the 16S ribosomal RNA (rRNA), the complete (tRNA)^{val} gene, and the 3' part of the 12S rRNA gene, with 110, 4, and 22 variable sites respectively (51, 2, 11 positions were potentially parsimony informative). Examination of base composition and base composition bias in the data sets are summarized in Table 3. Moderate amounts of bias were found in some of the components of the data sets. Due to this bias, a LogDet/paralinear transformation (Steel 1994, Lock-

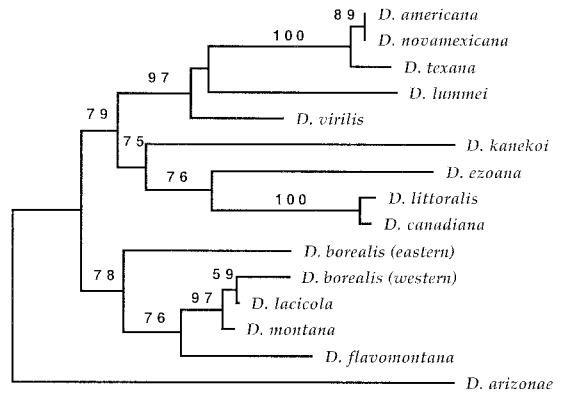


Fig. 2. Minimum evolution tree topology using LogDet/paralinear distance transformation. Values above the branches represent bootstrap percentages greater than 50%. The same tree was recovered when Jukes-Cantor and Kimura 2-parameter distances were used.

hart et al. 1994) of the data were also used to search for trees using the minimum evolution criterion (Saito and Imanishi 1989).

Phylogenetic Analyses of mtDNA. Parsimony analysis of the mtDNA data set resulted in a single minimum length tree of 194 steps, with a CI of 0.794 (0.664 excluding uninformative characters) and a RI of 0.775 (Fig. 1).

The searches under the minimum evolution criterion using the Jukes-Cantor (1969) ($L = 0.13935$), Kimura 2-parameter ($L = 0.13949$), or LogDet/paralinear ($L = 0.17308$) distances consistently recovered the same tree topology regardless of what model of sequence evolution was used (Fig. 2). This tree was one step longer than the parsimony tree and differed only in the placement of *D. kanekoi*.

The evaluation of the model resulted in the HKY 85 with Maximum likelihood resulted in an additional tree topology with a score of $-\ln = 2699.49493$ (Fig. 3). In this instance, the difference among topologies was the nonresolution among the phylads and subphylads, but with *D. kanekoi* related to the *D. littoralis*

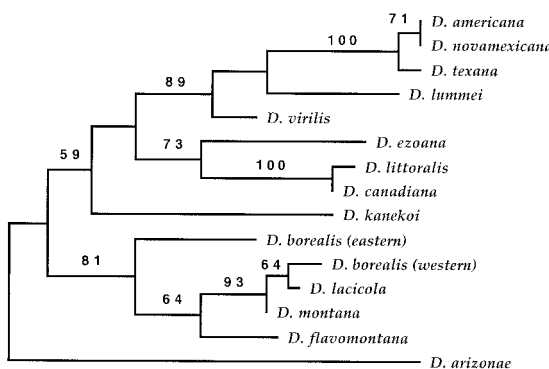


Fig. 1. Single most parsimonious tree topology for the mitochondrial 12S/16S rRNA data set. Values above the branches represent bootstrap percentages greater than 50%.

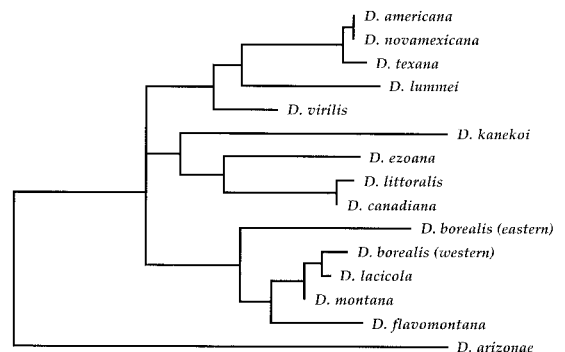


Fig. 3. Single tree uncovered under maximum likelihood search with a HKY85-71 model of sequence evolution. All parameters used in the model were estimated using maximum likelihood in PAUP*.

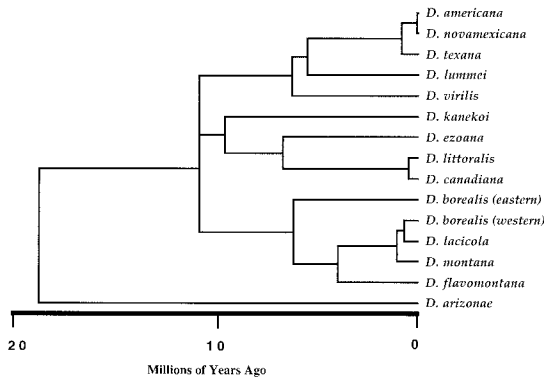


Fig. 4. Maximum likelihood tree topology with molecular clock enforced. The model is the HKY85 with the ti/tv ratio = 1.719445 and the $\alpha = 0.080126$ for the Γ distribution. The times of divergence have been estimated based on the assumption that the divergence of *D. melanogaster* from *D. yakuba* occurred eight million years ago. See text for explanation.

subphylad as in the distance tree. In addition, we failed to reject (Table 2) a molecular clock when it was enforced on this given topology (Fig. 4). Consequently, these data can be viewed as evolving in a relatively clock-like fashion.

Phylogenetic relationships within the *D. virilis* group. All tree topologies show support for four lineages within the *D. virilis* group, which have been previously reported (Spicer 1992); the virilis phylad (*D. virilis*, *D. americana* Spencer, *D. texana* Patterson, *D. novamexicana* Patterson, and *D. lummei* Hackman), the montana subphylad (*D. montana*, *D. borealis*, *D. laticola* Patterson, *D. flavomontana* Patterson) the littoralis subphylad (*D. littoralis*, *D. ezoana* Takada & Okada, and *D. canadiana*) and the kanekoi subphylad (*D. kanekoi*). Although there is strong support for these four lineages, there was little to no support for the relationships among these groups (Figs. 1-3).

One result consistent across all analyses was the separation of the two sampled strains of *D. borealis* that were included (*D. borealis*-eastern and *D. borealis*-western). These data suggest that *D. borealis* does not have a disjunct distribution, but that these strains are actually two separate cryptic species. These findings are consistent with other mitochondrial and nuclear DNA sequence data (G.S.S. and C.D.B., unpublished data).

The maximum likelihood divergence estimates for the *D. virilis* species allow an estimation of the divergence time among the species and thus provide a crude estimate of the maximum time required for an adaptive evolutionary change to occur. Currently, there is no biogeographic or fossil evidence for reliably dating the absolute divergence times of the species within the *D. virilis* species group, so we have therefore used the comparison of *D. yakuba* and *D. melanogaster* to calibrate our molecular clock. Biogeographic evidence indicates that these two species separated between 6 and 15 million years ago (Lachaise

et al. 1988), although the molecular evidence from ADH sequences suggests that the lower end of this range is probably more appropriate (Russo et al. 1995, Takezaki et al. 1995). Consequently, we have chosen a divergence value of eight million years ago (mya) for the separation of *D. yakuba* and *D. melanogaster*. By using the maximum likelihood molecular clock distance (model HKY85 + Γ , ti/tv ratio = 1.719445, $\alpha = 0.080126$) between *D. yakuba*/*D. melanogaster* we can infer a divergence of 0.00433/million years. Assuming that the 12S/16S rRNA sequences evolve at about the same rate in the *D. virilis* and *D. melanogaster* groups, we have used this rate to approximate a rough absolute molecular clock for the *D. virilis* species group.

Discussion

Phylogenetic hypotheses inferred from the parsimony, distance, and maximum likelihood searches of this data set resulted in tree topologies consistent with those found based on other data sets: chromosomal (Throckmorton 1982, Spicer 1992), two-dimensional electrophoresis (Spicer 1991), nuclear DNA sequences (Hilton and Hey 1996, 1997, Nurminsky et al. 1996) and mitochondrial DNA sequence data (G.S.S., unpublished data). The four subphylads that have been defined previously (Spicer 1992) are clearly resolved in all these analyses. However, the traditional division of the subgroup into two separate phylads (Throckmorton 1982) is not supported with this data set.

The phylogenetic relationships among taxa within phylads and subphylads are also confirmatory, for the most part. Relationships within the *D. virilis* phylad are different from those suggested by the reanalysis (Spicer 1992) of the electrophoretic data set of Throckmorton (1972 1978, 1982), but fully congruent with the chromosomal phylogeny (Spicer 1992) and the other molecular evidence (Spicer 1991, 1992, Hilton and Hey 1996, 1997, Nurminsky et al. 1996). Relationships within the other subphylads are less clear. As already mentioned, the *D. kanekoi* and *D. littoralis* subphylads are not considered as part of the *D. montana* phylad. However, once again, *D. kanekoi* appears to be a very distinctive and divergent species relative to the rest of the group, as our sequence data show and most other data sets have shown (Spicer 1992). The relationships within the *D. littoralis* subphylad are also what has been previously noted (Spicer 1992). In this case *D. littoralis* and *D. ezoana* form a monophyletic group, but are relatively distantly related. This is the first molecular study to examine the recently described species *D. canadiana* (Takada and Yoon 1989). Our results suggest that this species is valid, and although we cannot unequivocally state that it is a different species, it at least represents a very distinctive form of *D. littoralis* based on our sequence data.

Within the *D. montana* subphylad the relationships correspond to the findings of Spicer (1992), although with more resolution. As suggested by the reanalysis of Spicer (1992) and corroborated by Nurminsky et al.

(1996), *D. flavomontana* is the basal species in the group, which is confirmed by this data set with one exception. This exception concerns the divergence of *D. borealis*, which has revealed a dramatic divergence between the eastern and western populations of *D. borealis* (Fig. 4). It has long been noted that this species has a disjunct population (Patterson and Stone 1952), but it was not known if this was real, or simply a lack of collecting in the intermediate range (Throckmorton 1982). Our molecular results suggest that this is more than a fragmented population and probably represents two separate species. Given that *D. borealis* was described from western specimens originally collected in Idaho and Colorado, it appears that the eastern form currently represents an unnamed species. This result may help resolve some of the phylogenetic ambiguity of the *D. montana* subphylad, since many previous studies have lumped these two taxa together for analysis purposes (Throckmorton 1972, 1978, 1982; Spicer 1991).

The divergence times inferred by our analysis are also consistent with other studies. Ostrega (1985) suggested that the *D. virilis* and *D. montana* clades separated between 7 and 9 mya based on a restriction fragment study of mitochondrial genes. This estimate is in line with the ADH sequence study of Nurminsky et al. (1996) that inferred a divergence time between the *D. virilis* and *D. montana* groups at nine mya. Both of these sequence-based estimates are comparable to our estimate of 11 mya. However, these sequence estimates are lower than those for the protein-based studies. Reinbold and Collier (1990) reported an estimated divergence time between *D. virilis* and *D. montana* at ≈ 15 mya for an immunological study of 6-GPDH, whereas the two-dimensional protein electrophoretic study of Spicer (1991) suggested a divergence time of 20 mya for these two groups. Nevertheless, the relative divergence between groups is about the same. For example, the divergence between *D. virilis* and the other *D. virilis* phylad species is given as 2.6 mya by Nurminsky et al. (1996), while the same comparison by Spicer (1991) is given as 6.7 mya. The relative divergence between *D. virilis* and the other *D. virilis* phylad species to that of the divergence between the *D. virilis* and *D. montana* phylads is 28% for the Nurminsky et al. (1996) study (2./9.0), while the relative value for the study of Spicer (1991) is 33% (6.7/20.3). Consequently, it appears that the difference among studies in timing probably has more to do with how the absolute times of divergence were set, rather than any differences in the relative divergences among taxa in these groups. Unfortunately, setting the molecular clock for *Drosophila* taxa is still a difficult task that remains to be solved.

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