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Short Communication

# Molecular phylogeny of the *Drosophila tripunctata* and closely related species groups (Diptera: Drosophilidae)

Luciane Mendes Hatadani [a], James O. McInerney [b], Hermes Fonseca de Medeiros [a], Ana Carolina Martins Junqueira [c], Ana Maria de Azeredo-Espin [a,c], Louis Bernard Klaczko [a,*]

[a] Departamento de Genética e Evolução, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), P.O. Box 6109, Campinas, SP 13083-970, Brazil
[b] Bioinformatics Laboratory, Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland
[c] Laboratório de Genética Animal, Centro de Biologia Molecular e Engenharia Genética e Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), P.O. Box 6010, Campinas, SP 13083-875, Brazil

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ABSTRACT

We suggest a new phylogenetic hypothesis for the *tripunctata* radiation based on sequences of mitochondrial genes. Phylogenetic trees were reconstructed by parsimony, maximum likelihood and Bayesian methods. We performed tests for hypotheses of monophyly for taxonomic groups and other specific hypotheses. Results reject the monophyly for the *tripunctata* group whereas monophyly is not rejected for the *tripunctata* radiation and other specific groups within the radiation. Although most of the basal nodes were unresolved we were able to identify four clusters within the *tripunctata* radiation. These results suggest the collection of additional data before a proper taxonomic revision could be proposed.


## 1. Introduction

The *Drosophila tripunctata* species group is presently the second largest Neotropical group of *Drosophila* (surpassed by the *D. repleta* group), comprising 78 species according to the Taxodros database of May 2008 (Bächli, 2008). Frota-Pessoa (1954) subdivided the group into four subgroups (I–IV) based on morphological characters. The *D. tripunctata* group is almost endemic to the Neotropics (Throckmorton, 1975), where its species are abundant, particularly in forest areas, and a dominant component of the drosophilid fauna (Ashburner et al., 2005; Klaczko, 2006).

The *tripunctata* radiation was created by Throckmorton (1975) and included the *tripunctata* group, as well as other groups (*calloptera, cardini, guarani, macroptera, pallidipenis, rubrifrons* and *sticta*). According to this author, a radiation which he called *immigrans-Hirtodrosophila* originated in the Paleotropics, where it initially diversified and from where it sent two separate lineages to the Neotropics: *tripunctata* (composing the *tripunctata* radiation) and *Hirtodrosophila*. Throckmorton (1975) also suggested that the *tripunctata* group itself should not be considered a monophyletic group. This statement is in agreement with cytological observations (Kastritsis et al., 1970) and recent molecular studies (Yotoko et al., 2003; Robe et al., 2005). The monophyly of the *tripunctata* radiation as a whole has also been questioned (Remsen and O'Grady, 2002; Robe et al., 2005; Yotoko et al., 2003). In general, phylogenetic relationships among species belonging to the *tripunctata* radiation have been poorly studied, which has been pointed out by Markow and O'Grady (2006). Even though it seems clear that the *tripunctata* group is not monophyletic, the monophyly of the *tripunctata* radiation is still unresolved. Moreover, the studies mentioned previously were unable to recover a well supported phylogenetic hypothesis for relationships among the species groups within the radiation.

In this paper, we propose a new phylogenetic hypothesis for species of the *tripunctata* radiation of *Drosophila* based on sequences of mitochondrial genes of cytochrome oxidase subunits 1 and 2 (COI and COII) and test for different evolutionary hypotheses. Our aim was to improve the results obtained by Yotoko et al. (2003) and Robe et al. (2005) by adding both taxa and characters. In addition, we tested for monophyly of taxonomic groups (*calloptera*, *cardini*, *guarani* and *tripunctata*), of the *tripunctata* radiation, and of specific clades that appeared on the phylogenetic trees as monophyletic.

## 2. Materials and methods

### 2.1. COI and COII sequence data

Specific location of collection and taxonomic placement of each species are given in Table 1. All individuals included in the analysis

* Corresponding author. Fax: +55 19 3521 6235.
*E-mail address:* lbk@unicamp.br (L.B. Klaczko).

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**Table 1**
Taxonomic placement and collection site of each species collected for DNA extraction and accession numbers of all sequences included in the phylogenetic analyses.

| Subgenus | Group | Subgroup | Collection Site[a] | Species | Accession Number | |
|---|---|---|---|---|---|---|
| | | | | | COI | COII |
| *Drosophila* | *calloptera* | | Mata Ribeirão Cachoeira | *D. atrata* | EF569988 * | EF570024 * |
| | | | — | *D. ornatipenis* | EF570010 * | EF570038 * |
| | | | Bosque dos Jequitibás | *D. schildi* | EF570016 * | AY162973 |
| | *cardini* | | Serra do Japi | *D. cardini* | EF569991 * | AY162974 |
| | | | Brasilia | *D. cardinoides* | EF569992 * | AY162975 |
| | | | Bosque dos Jequitibás | *D. neocardini* | EF570006 * | EF570034 * |
| | | | Serra do Japi | *D. polymorpha* | EF570014 * | EF570040 * |
| | *guarani* | *guaramunu* | Serra do Japi | *D. griseolineata* | EF569995 * | EF570029 * |
| | | | Serra do Japi | *D. guaraja* | EF569996 * | EF570030 * |
| | | | Serra do Japi | *D. maculifrons* | EF569998 * | EF570031 * |
| | | guarani | Mata Ribeirão Cachoeira | *D. guaru* | EF569997 * | EF570031 * |
| | | | Serra do Japi | *D. ornatifrons* | EF570009 * | AY162978 |
| | *pallidipenis* | | Serra do Japi | *D. pallidipenis* | EF570011 * | AY162982 |
| | *sticta* | | Mata Ribeirão Cachoeira | *D. sticta* | EF570018 * | EF570044 * |
| | *tripunctata* | I | Mata Ribeirão Cachoeira | *D. nappae* | EF570005 * | AY162983 |
| | | | Mata Ribeirão Cachoeira | *D. neoguaramunu* | EF570007 * | EF570036 * |
| | | | Bosque dos Jequitibás | *D. setula* | EF570022 * | EF570042 * |
| | | | Serra do Japi | SP22[b] | EF570017 * | EF570043 * |
| | | II | Serra do Japi | *D. cuaso* | EF569993 * | EF570027 * |
| | | | Mata Ribeirão Cachoeira | *D. medioimpressa* | EF569999 * | AY162994 |
| | | | Serra do Japi | *D. mediopunctata* | EF570001 * | AY162988 |
| | | | Serra do Japi | *D. mediosignata* | EF570002 * | AY162985 |
| | | | Serra do Japi | *D. paraguayensis* | EF570012 * | EF570039 * |
| | | | Serra do Japi | *D. roehrae* | EF570015 * | EF570041 * |
| | | | Mata Ribeirão Cachoeira | *D. unipunctata* | EF570020 * | EF570047 * |
| | | III | Serra do Japi | *D. bandeirantorum* | EF569989 * | EF570025 * |
| | | | Bosque dos Jequitibás | *D. bifilum* | EF569990 * | EF570026 * |
| | | | Mata Ribeirão Cachoeira | *D. frotapessoai* | EF569994 * | EF570028 * |
| | | | Serra do Japi | *D. mediopicta* | EF570000 * | EF570033 * |
| | | | Serra do Japi | *D. mediostriata* | EF570003 * | EF570034 * |
| | | | Mata Ribeirão Cachoeira | *D. nigricincta* | EF570008 * | EF570037 * |
| | | | Serra do Japi | *D. paramediostriata* | EF570013 * | AY162995 |
| | | | Mata Ribeirão Cachoeira | *D. trifilum* | EF570019 * | EF570046 * |
| | | IV | Bosque dos Jequitibás | *D. metzii* | EF570004 * | AY162992 |
| | | | — | *D. tripunctata* | EF570023 * | AF519343 |
| | *quinaria* | | — | *D. falleni* | AY541136 | AF147117 |
| | | | — | *D. innubila* | AY541192 | AY541211 |
| | | | — | *D. quinaria* | AY154400 | AF478428 |
| | | | — | *D. recens* | AY154456 | AF147123 |
| | | | — | *D. subquinaria* | AY154457 | AY154457 |
| | *immigrans* | *immigrans* | Mata Ribeirão Cachoeira | *D. immigrans* | EF570021* | AY162993 |
| | *repleta* | *hydei* | — | *D. eohydei* | DQ471601 | AF145889 |
| | | | — | *D. hydei* | DQ471602 | DQ202020 |
| *Sophophora* | *melanogaster* | *melanogaster* | — | *D. melanogaster* | NC001709 | NC005779 |
| | | | — | *D. mauritiana* | NC005779 | NC001709 |
| | | | — | *D. sechellia* | NC005780 | NC005780 |
| | | | — | *D. simulans* | NC005781 | AF474082 |
| | | | — | *D. yakuba* | NC001322 | NC001322 |

[a] Coordinates for each collection site are: 22°55' S, 47°03' W (Bosque dos Jequitibás, Campinas, SP); 15°46 S 47°55 W (Brasília, DF); 22°50' S, 46°55' W (Mata Ribeirão Cachoeira, Campinas, SP); 23°13' S, 46°53' W (Serra do Japi, Jundiaí, SP).
[b] Undescribed species. SP22 and *D. nappae* are sibling species.
* New sequences obtained in this study.

were adult males, identified by the aedeagus, the most reliable method of identification of these species (Vilela, 1992). In addition, prior to DNA extraction, the terminalia of each male was removed and preserved in 70% alcohol. This procedure would allow for future confirmation of species identification and reevaluation in case of taxonomic revisions.

Total DNA of each individual was extracted using a phenol–chloroform protocol (Azeredo-Espin et al., 1991). The primers used for amplification were TL2-N-3014 and C1-J-2195 (COI), and TL-2-J3037 and TK-N-3785 (COII), described in Simon et al. (1994). The amplified products were purified with the QIAquick PCR purification kit. With the exceptions of the 5′ fragment of COI of *D. guaru, D. trifilum*, *D. maculifrons* and *D. setula,* and COII of *D. mediostriata*—in which case PCR products were cloned into the PCR2.1 cloning vector using a TA Cloning Kit (Invitrogen)—all PCR products were directly sequenced. Sequencing was performed using BigDye (Applied Biosystems) chemistry on either an ABI377A or an ABI3700 automatic sequencer. At least two sequences of each fragment were obtained for each individual to ensure high quality of sequences. Except for the cloned samples (three clones per species), for which we used primers provided by the Cloning Kit, the primers used for sequencing were the same as in PCR. All resulting sequence chromatograms were evaluated and edited with the use of the programs Phred (Ewing et al., 1998), Phrap and Consed (Gordon et al., 1998).

Additional sequences were obtained from GenBank whereas individuals of *D. ornatipenis* were obtained from Tucson Fly Stock

Center and *D. tripunctata* was kindly provided by Dr. Jean R. David. Accession numbers of all sequences are listed in Table 1.

For the analysis of COI, we obtained fragments of 1413 bp, whereas for the analysis of COII we obtained a fragment of 663 bp. The phylogenetic analyses included 48 taxa, of which 36 sequences of COI and 21 sequences of COII were newly obtained in this study (table 1).

Species from the *melanogaster* group were used as outgroup to root the trees. Other species included belonged to the *quinaria, immigrans* and *repleta* groups—in order to assess the monophyly of the *tripunctata* group and the *tripunctata* radiation.

### 2.2. Phylogenetic analysis and hypothesis testing

Sequences were aligned with ClustalW (Thompson et al., 1994), implemented as a tool in MEGA 3.1 (Kumar et al., 2004), followed by translation into amino acids for confirmation of alignment and assignment of codon positions.

PTP (permutation tail probability) tests were conducted to detect phylogenetic signal on the sequence data. Base composition heterogeneity among taxa was tested by $\chi^2$ test. Uncorrected distances were computed with PAUP (Swofford, 2003) in order to evaluate the amount of variation and homoplasy depending on codon position.

The maximum parsimony (MP) tree was obtained with PAUP, using TBR heuristic search (1000 repetitions with random taxon addition) whereas maximum likelihood (ML) trees were generated by Phyml (Guindon and Gascuel, 2003). In order to perform the ML analysis, we allowed the program to estimate both the proportion of invariant sites and the gamma distribution parameter, and base frequencies were estimated by maximum likelihood. Branch support for both MP and ML trees was computed using bootstrap resampling procedure (1000 replicates) and the results were summarized using a majority-rule consensus.

MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) was used to obtain the Bayesian tree. In order to account for differences in nucleotide substitution parameters in each codon position, we used a model partitioned by gene and codon position (Nylander et al., 2004). Posterior probabilities were based on two independent MCMC runs, each composed of four chains (three heated chains and one cold chain), with sample frequency of 1000 generations for a total of 70 million generations. We used a flat Dirichlet prior (non-informative) and the first 25% of the generations of each run was discarded as burn-in. Average standard deviation of split frequencies of the cold chain likelihoods between the two independent MCMC runs was used as convergence diagnostic.

The program Modeltest (Posada and Crandall, 1998) was used to select the best of the available models of nucleotide substitution for ML and Bayesian analyses.

All trees were reconstructed as unrooted trees and the five species belonging to the *melanogaster* group were later used to root the trees.

In order to test for specific hypotheses of monophyly we compared constrained and unconstrained trees. For each hypothesis, trees were reconstructed in which a constraint was used to enforce a specific group of taxa to be a monophyletic group. Tests of monophyly used were the Templeton (Wilcoxon-rank) and winning-sites tests on MP trees.

## 3. Results

### 3.1. COI and COII data description

The complete data set included 2076 bp, of which 1301 were constant, 97 were variable parsimony-uninformative and 678 were parsimony informative characters.

As expected, we found a low GC content for both genes (A, 30.3%; C, 15.2%; G, 15.3%; T, 39.2%), a common finding in insect mitochondrial sequences. No bias in base composition among taxa was detected by the $\chi^2$ test ($p$-value = 0.999) whereas the PTP test was significant ($p = 0.001$).

Considering all taxa, distances for all codon positions were 0.1168 ± 0.0005. When the third codon position was excluded distances were much lower (0.0278 ± 0.0002) than those obtained when second and first positions were excluded (0.2949 ± 0.0013), indicating a high level of homoplasy on the third codon position and a low amount of variation on the first and second positions.

### 3.2. Phylogenetic analysis

The model suggested by Modeltest with the Akaike information criterion (AIC) for the combined data was the Tamura–Nei model, with a gamma distribution of substitution rates across sites and proportion of invariant sites (TN93 + I + G). The TN93 + I + G model of substitution was used for the ML analysis and the GTR + I + G for the Bayesian reconstruction since it is not possible to specify the TN93 + I + G model of nucleotide substitution in MrBayes.

Maximum parsimony analysis resulted in only one most parsimonious tree (not shown), with 5358 steps (consistency index = 0.226; retention index = 0.396; homoplasy index = 0.774). The phylogenetic tree obtained by this method resulted, as expected, in the *repleta* and *immigrans* groups diverging first. The bootstrap 50% majority consensus tree resulted in a basal polytomy in the ingroup, with reliable support values only for the monophyly of the *cardini* group, three species of the *quinaria* group, and a few pairs of sibling species. This analysis per se does not allow for the discussion of hypotheses of monophyly of either the *tripunctata* radiation or the *tripunctata* group. As a consequence, the relationship among groups of the *tripunctata* radiation was also unresolved. Maximum likelihood reconstruction resulted in a tree very similar to the one obtained by MP (not shown), with analogous clusters.

In agreement with the two previous methods, Bayesian analysis revealed an early divergence of the *repleta* and *immigrans* groups (Fig. 1). The *quinaria* group diverges earlier than the remaining taxa, even though posterior probability for monophyly of the *tripunctata* radiation is low (0.71). A basal polytomy is observed within the *tripunctata* radiation, and clades with high posterior probabilities are: (1) a monophyletic *cardini* group (1.00); (2) a large clade composed mostly by species of the *tripunctata* group (0.97); (3) a clade composed by *guaramunu* subgroup, *tripunctata* and *calloptera* groups (0.98), within which a clade with high posterior probability (1.00) is formed by *D. griseolineata*, *D. maculifrons*, *D. frotapessoai* and *D. paramediostriata*; (4) there is also relatively high support (0.89) to a clade formed by *D. nappae*, SP22 (a pair of sibling species), and *D. setula*.

### 3.3. Tests of monophyly

We performed tests of the following specific hypotheses of monophyly of taxonomic groups:

i. *tripunctata* group
ii. *calloptera* group
iii. *cardini* group
iv. *guarani* group
v. *guaramunu* subgroup
vi. *quinaria* group
vii. *tripunctata* radiation

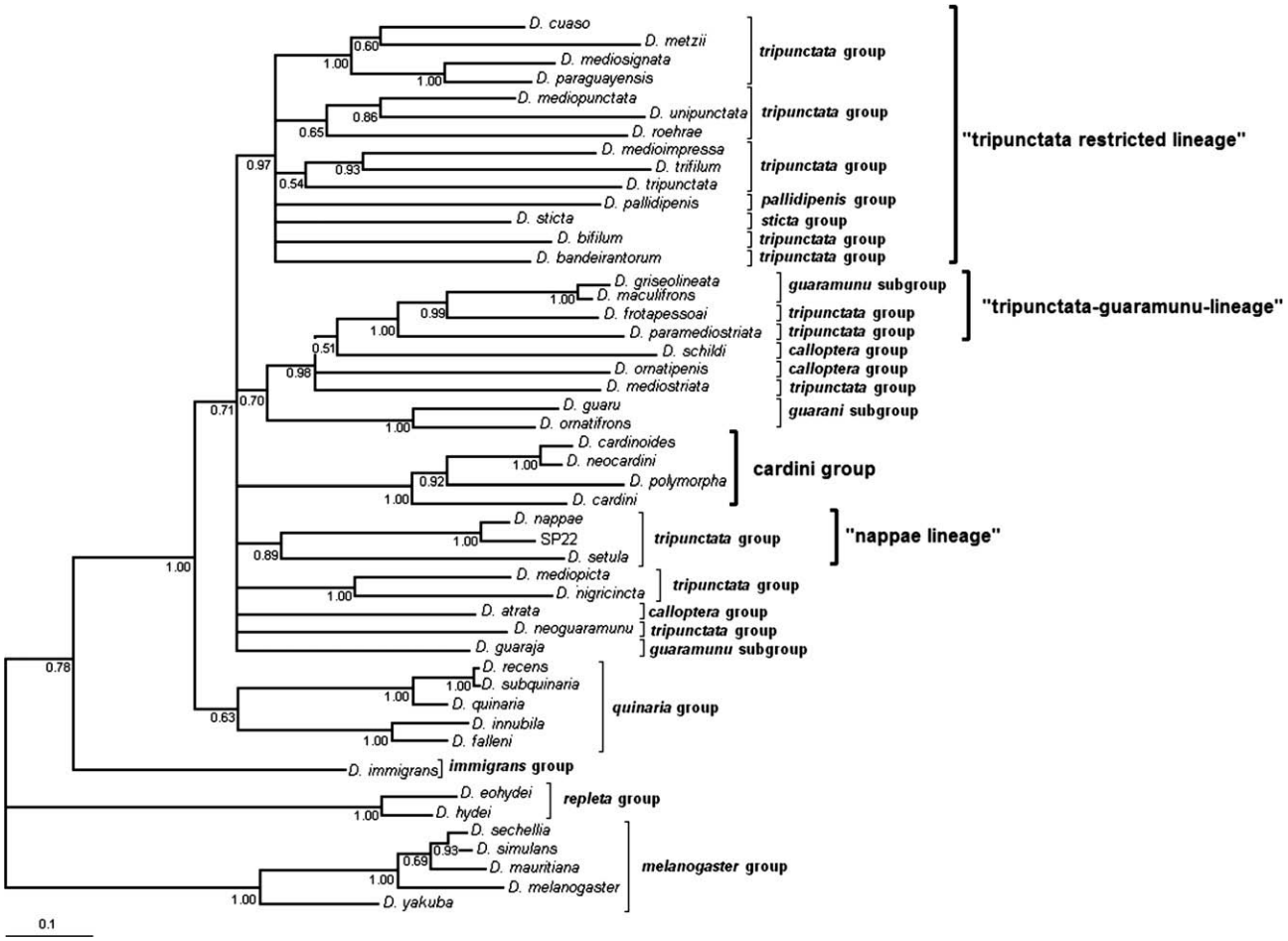


**Fig. 1.** Phylogenetic reconstruction by Bayesian analysis. Values below branches represent posterior probabilities. Branches were collapsed whenever posterior probabilities were lower than 0.50.

In addition, we also tested for the monophyly of the following clades:

viii. "*tripunctata* restricted lineage" (*D. bandeirantorum, D. bifilum, D. cuaso, D. medioimpressa, D. mediopicta, D. mediopunctata, D. mediosignata, D. metzii, D. pallidipenis, D. paraguayensis, D. roehrae, D. sticta, D. trifilum, D. tripunctata* and *D. unipunctata*)

ix. "*nappae* lineage" (*D. nappae*, *D. setula* and SP22)

x. "*tripunctata-guaramunu* lineage" (*D. mediostriata, D. paramediostriata, D. frotapessoai, D. maculifrons and D. griseolineata*)

xi. "*tripunctata-guaramunu* alternative lineage" (*D. paramediostriata, D. frotapessoai, D. maculifrons and D. griseolineata*)

xii. "*guarani* alternative lineage" (all species belonging to the *guarani* group, excluding *D. guaraja*).

The reason for testing the last hypothesis is that monophyly of the *guarani* group would consequently be rejected in case the monophyly of the *guaramunu* subgroup was also rejected due to *D. guaraja* not being closely related to *D. griseolineata* and *D. maculifrons*, according to our phylogenetic reconstructions.

Winning-sites and Templeton tests, comparing strict consensus trees produced according to each hypothesis, rejected monophyly for the *tripunctata* group ($p = 0.0107$ and $p = 0.0035$, respectively), for both constrained trees by the *guarani* group, either including or not *D. guaraja* ($p < 0.0001$ for both tests and both hypotheses) and for the "*tripunctata-guaramunu* alternative lineage" ($p < 0.0001$ for both tests). Monophyly of the *guaramunu* subgroup was also rejected by the winning-sites test ($p = 0.0387$) whereas this hypothesis was not rejected by the Templeton test ($p = 0.2065$).

## 4. Discussion

### 4.1. Phylogenetic analysis

All three methods of phylogenetic reconstruction (MP, ML and Bayesian) resulted, as expected, in the early divergence of the *D. repleta* and *D. immigrans* groups. Although the posterior probability was relatively low, the *tripunctata* radiation was recovered as monophyletic by Bayesian reconstruction. Moreover, winning-sites and Templeton tests did not reject monophyly for this clade. This result disagrees with the results obtained by Remsen and O'Grady (2002), and supports the hypothesis of monophyly of the *tripunctata* radiation, suggested earlier by Throckmorton (1975) and later reinforced by molecular studies (Yotoko et al., 2003 and Robe et al., 2005). On the other hand, our results did not recover a monophyletic *D. tripunctata* group, confirming previous suggestions (Throckmorton, 1975; Kastritsis et al., 1970; Yotoko et al., 2003; Robe et al., 2005).

Our results suggest the existence of four clusters within the *tripunctata* radiation: the *D. cardini* group and three clades which we called "*tripunctata* restricted lineage", "*nappae* lineage", and "*tri-*

*punctata-guaramunu* lineage". Even though MP and ML analyses resulted in low support values (with the exception of the *D. cardini* group), these groups were supported by posterior probabilities higher than 0.85 in the Bayesian reconstruction.

Brisson et al. (2006) analyzed sequence data of all 16 species belonging to the *cardini* group and obtained a tree for which strong support was given to the monophyly of *D. cardini* group as a whole. As expected, our trees also recovered a strongly supported monophyletic *D. cardini* group.

Kastritsis (1969) and Kastritsis et al. (1970) suggested that the *guarani* group should be split into two groups (*guarani* and *guaramunu*), based on polytene chromosomal banding analysis. He noticed that the chromosomes of species of the *D. guaramunu* subgroup were more similar to some of the *D. tripunctata* group than to those of the *D. guarani* subgroup, emphasizing the similarity between chromosomes of *D. griseolineata* and *D. mediostriata*. However, a recent molecular study by Robe et al. (2002) was unable to divide the *D. guarani* group into two subgroups. Our results recovered the relationships suggested by Kastritsis (1969) and Kastritsis et al. (1970): *D. griseolineata* and its sibling species *D. maculifrons* appear as closely related to the pair of sibling species *D. mediostriata* and *D. paramediostriata*, in addition to *D. frotapessoai*. One would expect *D. guaraja*—which also belongs to the *D. guaramunu* subgroup—to be found within this cluster. However, according to our results, its phylogenetic placement is uncertain and there is no evidence of a close relationship of *D. guaraja* and the cluster containing *guaramunu* subgroup species, which we named the *tripunctata-guaramunu* lineage. However, more inclusive phylogenetic studies are necessary before the controversy whether this group should be split into two (or more) groups is resolved.

The *D. calloptera* group is another group that should be further studied. Our Bayesian tree suggests a close relationship of species of the *guarani* group to *D. schildi* and *D. ornatipenis* whereas *D. atrata* is not placed within the same cluster. MP and ML methods place these three species in different positions, always with low support values. Therefore, our results were unable to determine phylogenetic positions of species of the *calloptera* group even though its monophyly (strongly supported by morphological evidence) was not rejected.

Even though the *D. tripunctata* group was not recovered as monophyletic in our study, we found a cluster with high posterior probability in which several species of this group were included in addition to *D. pallidipenis* and *D. sticta.* The remaining species placed in this cluster belong to subgroups II–IV, including the *paraguayensis* complex (composed by *D. paraguayensis, D. mediosignata and D. cuaso,* Bächli et al., 2000) and *D. metzii*; *D. roehrae* and *D. unipunctata* (sibling species); *D. mediopunctata*; *D. medioimpressa*; *D. trifilum*; *D. bifilum;* and *D. bandeirantorum*.

Our results suggest that the current taxonomic classification of the species of *Drosophila* belonging to the *tripunctata* radiation is incorrect regarding phylogenetic relationships. However, our results do not display enough resolution to propose any taxonomic revision of these groups. Both MP and ML methods of phylogenetic reconstruction resulted in trees with very low resolution, particularly for basal nodes. Bayesian reconstruction resulted in better support values than ML and MP but most of the basal nodes remain unresolved.

The pattern for the trees obtained by all three methods is the existence of short internal branches and long terminal branches, a pattern also observed by Yotoko et al. (2003). This kind of branching pattern is often interpreted as evidence of periods of rapid speciation. This hypothesis has been suggested by Throckmorton (1975) but it is difficult to confirm, since a polytomy on a gene tree does not necessarily correspond to a polytomy on the species tree (Slowinski, 2001). This is due to differences between gene trees and species trees (Pamilo and Nei, 1988; Doyle, 1997). Most tests designed to detect polytomies do not make this distinction and therefore are not useful for testing the hypothesis of rapid speciation in the *tripunctata* radiation (see examples in Slowinski, 2001). Slowinski (2001) proposed a test for the detection of species polytomies. However, this test requires independently inherited gene trees. As both genes analyzed in this study were mitochondrial genes, and therefore not independently inherited, this test cannot be applied unless more data (nuclear sequence data) is collected. In addition, our data revealed very low variation on the first and second codon positions and high homoplasy on the third codon position and that could be causing deeper relationships to be more difficult to recover. Jian et al. (2008) suggested that with enough data, rapid radiations could be resolved. On the other hand, Kolaczkowski and Thornton (2007) suggested that under realistic conditions even extremely long sequences are not enough to prevent frequent inference of strong support for incorrect clades. Nonetheless, perhaps the analysis of a larger data set, particularly including nuclear genes—with more appropriate nucleotide substitution rates—could be useful to resolve basal nodes within the tripunctata radiation and lead to consistent conclusions about the patterns of speciation of the *D. tripunctata* radiation.

Our work may motivate future phylogenetic studies on these species and a possible taxonomic revision, which appears to be necessary since we can now state with reasonable confidence that the *tripunctata* group is not monophyletic, as probably some other groups belonging to the *tripunctata* radiation. The collection of additional molecular data (preferably nuclear genes) is obligatory before we can establish a reliable phylogenetic hypothesis or conclude that the *tripunctata* radiation in fact originated from episodes of rapid or multiple speciation.

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