RESEARCH ARTICLE

Phylogeny of Drosophila saltans group (Diptera: Drosophilidae) based on morphological and molecular evidence

Bruna Emilia Roman ID1*, Diego J. Santana ID2, Carolina Prediger1, Lilian Madi-Ravazzi ID1*

1 Department of Biology, Institute of Biosciences, Humanities and Exact Sciences, São Paulo State University (UNESP), São José do Rio Preto, São Paulo, Brazil, 2 Biosciences Institute, Federal University of Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul, Brazil

* brunaemiliar@gmail.com (BER); lilian.madi@unesp.br (LMR)

Abstract

Drosophila saltans group belongs to the subgenus Sophophora (family Drosophilidae), and it is subdivided into five subgroups, with 23 species. The species in this group are widely distributed in the Americas, primarily in the Neotropics. In the literature, the phylogenetic reconstruction of this group has been performed with various markers, but many inconsistencies remain. Here, we present a phylogenetic reconstruction of the saltans group with a greater number of species, 16 species, which is the most complete to date for the saltans group and includes all subgroups, in a combined analysis with morphological and molecular markers. We incorporated 48 morphological characters of male terminalia, the highest number used to date, and molecular markers based on mitochondrial genes COI and COII. Based on the results, which have recovered the five subgroups as distinct lineages, we propose a new hypothesis regarding the phylogenetic relationships among the subgroups of the saltans group. The relationships of the species within the sturtevanti and elliptica subgroups were well supported. The saltans subgroup showed several polytomies, but the relationship between the sibling species D. austrosaltans and D. saltans and their close relation with D. nigrosaltans were well supported in the molecular and total evidence analyses. The morphological analysis additionally supported the formation of the clade D. nigrosaltans—D. pseudosaltans. The observed polytomies may represent synchronous radiations or have resulted from speciation rates that have been too fast relative to the pace of substitution accumulation.

Introduction

Phylogenetic reconstructions based on integrative analyses of different sets of characteristics (e.g., molecular and morphological characters) enable us to deduce robust evolutionary hypotheses [1, 2]. In addition, approaches that use several lines of evidence can reconstruct better relationships among taxa, mainly within groups with historical problems [3, 4]. Although molecular methods have more often been used to infer phylogenetic relationships
between organisms, the use of nonmolecular data is still highly recommended for identifying synapomorphies [5]. Morphological data are thus fundamental for decision-making in taxonomy and systematics [6]. Considering the genus Drosophila, the use of adult terminalia characters is particularly useful because they are the least homoplastic [7]. Among the various groups that need more robust phylogenetic evaluation, Drosophila species from the Neotropical region need a more complete and better supported phylogenetic hypothesis [8].

Drosophila saltans group is nested within the genus Drosophila and subgenus Sophophora and is closely related to the willistoni sister clade in the New World Drosophila diversification [9]. The geographical distribution of species in the saltans group extends across the entire region of Mexico to the state of Rio Grande do Sul in southern Brazil [10–13]. The parasaltans and cordata subgroups occur only in the Neotropical region, while some species of the elliptica, sturtevanti and saltans subgroups occur in the Neotropical and Nearctic regions [10].

Historically, the saltans group was first divided into two subgroups [14], but this division was later modified by Pavan and Magalhães [15] and Magalhães [16]. After that, Magalhães and Björnberg [17] divided the group into five subgroups, without naming, based on morphological characters with an emphasis on male terminalia. Then, with a total of 19 species, the five subgroups were named saltans (D. saltans, D. austrosaltans, D. lusaltans, D. prosaltans, D. nigrosaltans, D. pseudosaltans and D. septentriosaltans), sturtevanti (D. sturtevanti, D. milleri and D. rectangularis), parasaltans (D. parasaltans, D. subsaltans and D. pulchella), elliptica (D. elliptica, D. emarginata, D. neelliptica and D. neosaltans) and cordata (D. cordata and D. neocordata) [10]. However, Vilela and Bächli [18] observed the lectotype of D. pulchella and inserted this species into the sturtevanti subgroup and even suggested it as a synonym to D. sturtevanti. Mourão and Bicudo [11] added two new species to the sturtevanti subgroup (D. dacunhai and D. magalhaesi). Recently, Guillín and Rafael [12] introduced the species D. neo-prosaltans in this group, and Madi-Ravazzi et al. [19] included another new species (D. lehmannae) in the sturtevanti subgroup. Throughout these investigations, the number of species belonging to the saltans group increased to 23 [20].

Many studies have discussed the phylogeny of the saltans group [21–29]. However, some were more relevant regarding the robustness of the analysis and support of the trees. O’Grady et al. [24] performed the first molecular phylogeny with nine species of the saltans group. According to their total evidence tree, the cordata subgroup would have branched off the earliest, followed by the elliptica subgroup (D. emarginata), and the sturtevanti subgroup (D. milleri and D. sturtevanti) was recovered as the sister of the saltans-parasaltans clade. However, the relationships among species of the saltans subgroup were not well resolved, suggesting a recent divergence [24]. Moreover, Rodríguez-Trelles et al. [25] also proposed a phylogeny of the saltans group based on analyses of some molecular markers including xanthine dehydrogenase (Xdh). One of the trees generated with robust support showed the parasaltans subgroup (D. subsaltans) as sister to all others, followed by branching off the sturtevanti (D. sturtevanti), elliptica (D. emarginata), cordata (D. neocordata) and saltans (D. saltans and D. prosaltans) subgroups. Thus, the differences in marker genes and ingroup taxon-sampling between these studies would have affected the phylogenetic inference, resulting in topologies differing in the basal branch.

Morphological characters have also been fundamental tools in the delineation of the saltans phylogeny. Yassin [28] inferred the phylogeny of this group by coding 40 morphological characters from different life stages and of external and internal morphology. The generated tree supported the sturtevanti subgroup (D. sturtevanti and D. milleri) as the sister of all others in the group. The other subgroups formed two clades, one consisting of the cordata (D. neocordata) and elliptica (D. emarginata) subgroups and the other consisting of the parasaltans (D. subsaltans) and saltans (D. saltans, D. prosaltans, D. austrosaltans and D. lusaltans) subgroups.
Again, the relationships between *saltans* subgroup species were not resolved [28]. Last, Souza et al. [29] used morphological data from male terminalia to infer the phylogeny of the group. This work demonstrated the *cordata* (*D. neocordata*) subgroup as the sister taxon of all others and the formation of two large clades, one consisting of the *elliptica* (*D. emarginata*) and *sturtevanti* (*D. sturtevanti*, *D. dacunhai* and *D. milleri*) subgroups and the other of the *parasaltans* (*D. parasaltans*) and *saltans* (*D. saltans*, *D. prosaltans*, *D. lusaltans* and *D. austrosaltans*) subgroups [29].

The studies mentioned above used a limited number of taxa and few male terminalia characters, which are the most variable even among closely related species and mainly used to distinguish species in insect taxonomy, in their phylogenetic reconstructions, resulting in inconsistencies mainly for the *saltans* subgroup. Yassin [28] analyzed the inconsistencies of molecular phylogenetic inferences for the *saltans* group and pointed out that codon usage bias (CUB) may be an issue in this clade because nonstationarity and nonhomogeneity of the nucleotide composition can distort phylogenetic inferences, when compositional changes do not occur according to the genealogy of the species. Indeed, the Neotropical *Sophophora* (i.e., the *saltans* and *willistoni* groups) have higher frequencies of adenine and thymine at the third position of the code of their nuclear genome [25, 30–32]. A study of the nuclear and mitochondrial CUB patterns in other insects pointed out that the mitochondrial genome has higher CUB [33]. Therefore, we present a new hypothesis for phylogenetic relationships among subgroups of the *saltans* group based mainly on a total evidence dataset (morphological and molecular markers) and a greater number of species (16 species) than that already employed. In addition, we explored some difficulties found in the phylogenetic reconstruction of this group, focusing on the *saltans* subgroup.

**Materials and methods**

**Taxon sampling**

In the present study, 16 of the 23 species of the *saltans* group were evaluated based on morphological and molecular data. The species, strains and geographical origin are listed in S1 Table.

**Terminalia preparation and morphological characters**

The structures of terminalia were dissected and mounted based on Kaneshiro’s [34] technique. The distal two-thirds of the abdomen of each fly was extracted by stylets and placed in a microtube containing 10% KOH solution for 15 minutes in a water bath; then, the structures were transferred to a microtube containing a drop of eugenol and incubated for 24 h. After that, the terminalia was dissected with the aid of a stereomicroscope and stylets. The structures were dehydrated with pure acetone and mounted on stubs with copper tape for adhesion and electron conductivity [29]. The samples were sputter coated with gold in and analyzed by scanning electron microscopy (LEO 435 VPi Zeiss).

Based on the descriptions and analysis of the male terminalia electron micrographs by Roman and Madi-Ravazzi [35], 48 morphological characters were collected, and a matrix was coded in absence (0), presence (1) and presence with modifications (2) (Table 1).

**DNA sequencing**

The genomic DNA of males of the species studied was extracted from the whole body and by individual maceration using a Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) following the manufacturer’s protocol.
Table 1. Matrix of 48 coded morphological characters.

| Character | D. austrosaltans | D. lusaltans | D. nigrosaltans | D. prosaltans | D. saltans | D. septentriosaltans | D. dacunhai | D. milleri | D. sturtevanti | D. lehrmanae | D. neosaltans | D. neocordata | D. parasaltans | D. willistoni |
|-----------|------------------|--------------|-----------------|---------------|------------|---------------------|-------------|-----------|---------------|-------------|-------------|------------|---------------|------------|
| 0. Epandrial Ventral Process | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 |
| 1. Semi-elliptical shaped surstylus | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2. Hand-shaped surstylus | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 3. Elongated surstylus | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| 4. Surstylar teeth arranged throughout the intern portion of the surstylus | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| 5. Surstylar process | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| 6. Elongated hypandrium | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 2 | 2 | 2 | 0 | 1 | 0 |
| 7. Median gonocoxites rounded and convergent | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8. Long hypandrium | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| 9. Sickle-shaped aedegus | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| 10. Pregonites fused to the end | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| 11. Pregonites fused into a single structure | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 12. Long and bilateral ventral postgonites | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| 13. Aedegus apex with punctiform projection | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| 14. Short punctiform projection at the aedegus apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15. Bipartite aedegus apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| 16. Cylindrical aedegus apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 17. Membraneous aedegus apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 18. Ventral region of aedegus apex with a pair of chitin en hooks | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 19. Apical crest covered with scales at the aedegus apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 20. Grooves at the aedegus apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 21. Bristles at the aedegus apex | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22. Scales at the aedegus apex | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 23. Elongated and curved back aedegus apex | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 24. Aedegus sheath | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25. Smooth aedegus sheath with serrated edge | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 26. Scales-like structures in the dorsal region of aedegus sheath | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 27. Serration on the aedegus sheath | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28. Ventral postgonites | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| 29. Aedegus with only one ventral postgonite | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| 30. A pair of ventral postgonites | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 31. A pair of bifurcate ventral postgonites | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 32. Scales in the upper ventral region of aedegus | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
Table 1. (Continued)

|   | D. austrosaltans | D. baumans | D. nigroalbus | D. prosaltans | D. pseudosaltans | D. saltans | D. septentriosaltans | D. dacuchai | D. milleri | D. sturtevanti | D. lehrmanae | D. emarginata | D. neoelliptica | D. naralans | D. neocordata | D. parasaltans | D. willistoni |
|---|-----------------|------------|---------------|---------------|-----------------|-----------|---------------------|------------|----------|-------------|-------------|---------------|--------------|-------------|-------------|---------------|---------------|
| 33. Scales at the ventral postgonite | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 34. Enlarged ventral postgonite | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 35. Groove in the upper portion of ventral postgonite | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 36. Thin ventral postgonite without scales | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 37. Ventral postgonite slightly curved | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 38. Ventral postgonite combine with aedeagal apex result in a V-shaped | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 39. Ventral postgonite combine with aedeagal apex result in a C-shaped | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 40. A pair of lateral postgonites | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 41. A pair of aedeagal ventral process | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 42. Aedeagal ventral process bifurcated at the end | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 43. Enlarged and darkened aedeagal ventral process | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 44. Aedeagal ventral crest | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 45. Pair of aedeagal ventral crest | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| 46. Cleft on the dorsal region of aedeagus | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 47. A pair of long protuberances arranged laterally and fused to the aedeagus | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |

0 = absence, 1 = presence and 2 = presence with modifications.

[https://doi.org/10.1371/journal.pone.0266710.t001](https://doi.org/10.1371/journal.pone.0266710.t001)
The mitochondrial genes were amplified through polymerase chain reaction (PCR); the primer sequences and PCR annealing temperatures are shown in S2 Table. The amplification conditions were the same as in Madi-Ravazzi et al. [19]. Amplicons were sequenced by the Sanger method at Centro de Recursos Biológicos e Biologia Genômica (CREBIO). All generated sequences were deposited in GenBank (S1 Table).

**Parsimony analysis of morphological data**

The analysis based on the maximum parsimony criterion was performed using the TNT [36] and Winclada software was used to visualize and edit trees [37]. The analysis was performed using heuristic searches by the traditional method: starting with the construction of Wagner trees and refining by branch swapping in the tree-bisection-reconnection (TBR) algorithm, with 1000 replicates and retaining 10 trees per replicate. The characters were analyzed with equal weights. A strict consensus was reached for the most parsimonious trees resulting. Branch support was estimated using bootstrap (BS) analysis [38] (significance values ≥ 70%), calculated with 1000 replicates, using the heuristic search and the traditional search methods.

**Molecular phylogenetic analysis**

The editing of the sequences of the COI and COII genes as well as the comparison and creation of the consensus sequences of the forward and reverse sequences of each taxon were performed manually in BioEdit 7.2.5 [39], where they were also multiple aligned using ClustalW [40].

For phylogenetic analyses, we included COI and COII sequences (totaling 1,354 bp) from 16 species of the saltans group and D. willistoni as the outgroup (S1 Table). We determined the model of nucleotide substitution for each gene and the concatenated sequence datasets with jModelTest [41] using the Bayesian Information Criterion. The best-fit models were GTR+G. We performed a Bayesian phylogenetic analysis for the concatenated mitochondrial sequences (COI and COII) with MrBayes v3.2 [42]. We ran 100 million generations, sampling every 10,000 steps by using a Yule process tree prior. We checked for stationarity by visually inspecting trace plots and ensuring that all values for effective sample size (ESS) were above 200 in Tracer v.1.5 [43]. The first 25% of sampled genealogies were discarded as burn-in.

**Total-evidence analysis**

We performed a total evidence analysis with both morphological and molecular data using partitioned Bayesian analyses of total evidence implemented in MrBayes v3.2 [42] to infer the phylogenetic relationships within the saltans group. This analysis assumes different models (e.g., θa, θb, 0c) for different sets of partitions (e.g., Xa, Xb, Xc). The substitution models for COI (GTR+G) and COII (GTR+G) were tested in jModelTest [41] using the Bayesian information criterion. We used a gamma distribution for the morphological data [44]. We ran 100 million generations, sampling every 10,000 generations. We determined stationarity and ensured that all ESS values were 200 by using Tracer. The first 25% of sampled genealogies were discarded as burn-in.

**Codon usage bias**

The GC content of the third codon position (GC3) and the relative synonymous codon usage (RSCU) for the concatenated sequences of COI and COII were calculated using the R package seqinR [45] and DNAsp [46], respectively. To evaluate the heterogeneity of the CUB for the saltans group, disparity index test [47] and composition distance were calculated in MegaX.
Two clustering methods were implemented using the averages RSCU: a hierarchical clustering generated by heatmap and a correspondence analysis were implemented in the R packages ggplot2 [49] and FactorMineR [50], respectively. In correspondence analysis, codons that were not seen for all species were removed (S3 Table).

Results

Out of the 48 morphological characters coded, 29 were parsimony informative (i.e., shared by multiple species of the ingroup). The analysis resulted in eight equally parsimonious trees, and from these, the strict consensus was calculated (Fig 1A). The relationship among the subgroups was not resolved due to a polytomy. However, the species were grouped into the five subgroups [20]. The species of the sturtevanti subgroup were grouped in a clade with high support (BS = 96%) and supported by three synapomorphies: aedeagal apex with punctiform projection, aedeagus with only one ventral postgonite and scales in the upper ventral region of aedeagus. The species of the elliptica subgroup were grouped into one clade (BS = 80%), supported by synapomorphic characters of elongated surstylus and hypandrium and the presence of a pair of lateral postgonites. Within this clade, D. emarginata and D. neoeelliptica formed an internal clade (BS = 82%). Although the saltans subgroup was recovered as a monophyletic group (BS = 75%), relationships among component species were less resolved, being left in a polytomy except for the sister relationship between D. nigrosaltans and D. pseudosaltans (BS = 88%). In this analysis, the cordata and parasaltans subgroups were each represented by a
single species, *D. neocordata* and *D. parasaltans*, respectively. They were placed as independent branches with five and three autapomorphies, respectively: *D. neocordata* characterized by the presence of surstylus process, long and bifurcate ventral postgonites, pregonites basally fused into a single structure, frontal region of aedeagal apex with a pair of chitinous hooks, and a pair of long protuberances arranged laterally and fused to the aedeagus; and *D. parasaltans* by the presence of hand-shaped surstylus, membranous aedeagal apex, and the presence of a pair of bifurcate ventral postgonites (Table 1).

On the other hand, the Bayesian analyses with the concatenated mitochondrial-sequence dataset and Total Evidence dataset (morphological and molecular data) generated very similar trees resolving relationships among the subgroups with high supports (Figs 1B and 2). The

---

**Fig 2.** Phylogenetic relationships among species of the *saltans* group inferred by the total evidence analysis with morphological and molecular data. Bayesian posterior probability (PP) is shown as percentage on each node. The phallus of each species is shown in profile on the same scale. The letters represent the phallus of the following species: A = *D. emarginata*, B = *D. neoelliptica*, C = *D. neosaltans*, D = *D. neocordata*, E = *D. dacunhai*, F = *D. milleri*, G = *D. sturtevanti*, H = *D. lehrmanae*, I = *D. lusaltans*, J = *D. prosaltans*, K = *D. septentriosaltans*, L = *D. pseudosaltans*, M = *D. nigrosaltans*, N = *D. austrosaltans*, O = *D. saltans*, P = *D. parasaltans*, Q = *D. willistoni*.

https://doi.org/10.1371/journal.pone.0266710.g002
Parasaltans subgroup (represented by D. parasaltans) was placed as the sister to all the others in the saltans group (PP = 100%). The saltans subgroup was recovered as monophyletic (PP = 100%) and sister to a large clade consisting of the other three subgroups (PP = 100% for the mitochondrial dataset and PP = 97% for the Total Evidence dataset). Within the saltans subgroup clade, relationships among component species were less resolved, except for the close relationships ((D. saltans, D. austrosaltans), D. nigrosaltans) supported with PPs = 100%. Relationships among the three subgroups within the remaining clade were inferred as follows: [the elliptica + cordata subgroups (PP = 59% for the mitochondrial dataset and 75% for the Total Evidence dataset)] + [the sturtevanti subgroup (PP = 93%)]. Relationships among component species of the elliptica and sturtevanti subgroups were fully resolved: [[D. emarginata + D. neoelliptica (PP = 100%)] + [D. neosaltans (PP = 100%)]] in the elliptica subgroup; and [[D. dacunhai + D. milleri (PP = 100%)] + [D. sturtevanti + D. lehrmanae (PP = 97% for mitochondrial dataset and PP = 99% for Total Evidence dataset)]] in the sturtevanti subgroup.

The pairwise comparison of disparity index test and the composition distance indicated that the composition of D. neosaltans (elliptica subgroup) is significantly heterogeneous. Despite the conservative nature of the applied method, the null hypothesis that sequences have evolved with the same pattern of substitution for all the nucleotides was rejected in approximately 75% of the pairwise comparisons between D. neosaltans and the other species (S4 Table). The base composition bias by site found was higher for this species, especially in the third codon positions (S5 Table).

Aiming to verify the codon usage bias for the concatenated mitochondrial sequences of COI and COII, we calculated the GC content of the third codon position (GC3) for each species. Those results indicate that the mitochondrial region (COI and COII) is AT3 rich (varying from 89.4 to 98.5%; Fig 3A). The elliptica subgroup presented the highest GC3 (from 8.20 to 10.64%). The GC3 content varied most within the sturtevanti subgroup (from 2.00 to 7.10%), which the species D. sturtevanti and D. lehrmanae presented higher values of GC3 (5.3 and 7.1%) than D. dacunhai and D. milleri (2.9% and 2%, respectively). Within the saltans subgroup, the higher GC3 content was seen for the D. saltans (5.3%) and were particularly low for D. lusaltans (1.6%). Interestingly, the insular species (D. lusaltans and D. milleri) presented the lowest GC3. Through the heatmap the saltans and sturtevanti subgroups were recovered, while the elliptica subgroup was not because D. neosaltans was clustered with D. neocordata and D. parasaltans (Fig 3B). Similarly, the correspondence analysis also clustered of the saltans and sturtevanti subgroups (Fig 3C), which were separated by the first dimension of this analysis (see S1 Fig to check the contribution of each codon in each dimension). In the elliptica subgroup, the three species do not show similarity in the use of codons, which contributed the most to the first dimension, however considering the second dimension, D. neoelliptica and D. emarginata are very similar whereas D. neosaltans codon usage looks like D. neocordata and D. parasaltans (Fig 3C).

Discussion

The combination of morphological and molecular markers proved to be very important for unraveling relationships among species within the saltans group and resulted in a robust phylogeny. In the analyses performed here, the studied species were classified into five lineages corresponding to the established subgroups [20], suggesting the reliability of the selected markers. Drosophila parasaltans is the only representative, in the present study, of the parasaltans subgroup and was recovered as a sister taxon of all the others in the saltans group, with high support, in the analysis of total evidence and molecules, corroborating with data from the literature [25, 51].
In our analysis with molecular markers, as well as in the analysis of total evidence, the *sturtevanti* subgroup is sister of *cordata-elliptica* clade. The close relationship between the *cordata* and *elliptica* subgroups corroborates several studies [24, 25, 28, 51]. It is interesting to demonstrate that in addition to the phylogenetic works mentioned above, the *cordata-elliptica* relationship was observed by Castro and Carareto [27] in a study with the P family of elements, in which the authors found a very rudimentary and divergent sequence of the same element in each *D. neocordata* (*cordata* subgroup) and *D. emarginata* (*elliptica* subgroup) species, suggesting proximity between these species and consequently between these subgroups.
The relationship among the species of *elliptica* subgroup was strongly supported in all analyses, and it was grouped in the same way, establishing two internal clades: one composed of *D. emarginata* and *D. neoelliptica* and another clade composed of *D. neosaltans*, close to the previous two. This is the first time that a phylogenetic study has been carried out with three of the four species included in this subgroup; other studies have only been performed with *D. emarginata* [24, 25, 28, 29, 51]. It is interesting to note that the relationships seen in the phylogenetic tree can also be observed in the morphological characters of these species, where the aedeagi of *D. emarginata* and *D. neoelliptica* are extremely similar, differing markedly from *D. neosaltans* [35]. The most striking features are found in the size and shape of aedeagus, in which aedeagus of *D. neosaltans* is smaller (~16% of body size, whereas for *D. emarginata* it is ~50% and *D. neoelliptica* ~30%) and has no sickle shape, the aedeagal apex is cylindrical and not hooked and forked and the pregonites are not fused to the end (Fig 2C) [35]. Furthermore, *D. neosaltans* differs from the other two species because apparently it does not present the structure of the epandrial ventral processes, and in the same place, it has only two small saliences, suggesting that this structure may be present in the ancestral species of the subgroup [35].

The relationships of species within the *sturtevanti* subgroup were recovered with good support. In all analyses, the species were similarly grouped, establishing two internal clades, one composed of *D. sturtevanti* and *D. lehrmanae* and the other composed of *D. dacunhai* and *D. milleri*. This cluster structure was also observed in the GC3 analysis, and it was observed by Madi-Ravazzi et al. [19], who analyzed the same species with four mitochondrial markers, and by Souza et al. [29], who used morphological characteristics without *D. lehrmanae* species. This subdivision can be visualized through the morphology, shape and size of the four aedeagus species, which are very similar, but there is a greater similarity of structures between *D. dacunhai* and *D. milleri*, such as the presence of pointed scales on the ventral postgonite and a groove in the upper part of it, whereas *D. sturtevanti* and *D. lehrmanae* present this structure without scales, smooth and thin (Fig 2E–2H) [35].

Furthermore, studies on reproductive isolation with four species (*D. dacunhai*, *D. magalhaesi*, *D. milleri* and *D. sturtevanti*) showed several degrees of isolation, from complete isolations to fertile crosses, and the presence of inseminated females in several crosses that did not produce progeny was observed, suggesting that these results may be related to the courtship behaviors and the similarity among the morphologies of the aedeagi [11, 19, 23]. So, although there is species specificity of male terminalia, the morphology of male terminalia among species in the *sturtevanti* subgroup is generally very similar, unlike what is seen in species in other subgroups of the *saltans* group. The question of why this similarity exists is unanswered.

The reconstruction of evolutionary relationships of the *saltans* subgroup has been considered particularly challenging, and inconsistencies have been reported for different molecular markers [24, 25], which were not solved by the analysis of morphological characters [28, 29]. To solve this problem, we analyzed all species of this subgroup; however, only the establishment of the sibling species *D. austrosaltans* and *D. saltans* resulting from the molecular and total evidence analyses of the present work was recovered. This last relationship is corroborated in studies by Bicudo [22] and Nascimento and Bicudo [52] carried out with chromosomal inversion and pattern of esterases, respectively. In addition, the morphological analysis showed that only the relationship between *D. nigrosaltans* and *D. pseudosaltans* was robustly recovered. This information is new in the *saltans* subgroup phylogeny, but it can be easily observed in the morphological characteristics of the aedeagus, as both species present elongated and curved back aedeagal apex, different from other species (Fig 2L and 2M) [35].

Previous studies found different topologies and polytomies for the *saltans* subgroup, which is considered the most recent divergence of the *saltans* group, having occurred approximately 4.5 million years ago [9, 24, 28]. Phylogenetic reconstruction among species of recent
divergence can become much more complex due to three biological problems: segregation of polymorphisms that predate species divergence (incomplete lineage sorting), gene flow during and after speciation and intralocus recombination (hybridization) [53]. In fact, many recent studies have shown that these processes play an important role in the evolution of many taxa [54–56]. Gene flow among saltans subgroup species may still occur, and the species may continue to hybridize even at low frequencies [21]. This last study carried out a reproductive isolation experiment with seven species of the saltans subgroup and observed variable results, in which crosses between geographically distant species showed the production of fertile hybrids, even at low frequency [21]. Furthermore, in studies of chromosomal polymorphisms with this subgroup, Bicudo [22] found a common karyotype and a considerable degree of sequential homology for all species of the saltans subgroup. In addition, many other issues may lead to difficulties in constructing its phylogeny, such as rapid speciation rates relative to substitution rates, and heterogeneity in nucleotide composition biases [24, 28, 30, 31].

The codon usage bias analysis performed for the entire saltans group recovered low GC3 content in agreement with literature previews studies [57], which characterized the mitochondrial genes COI, II and III as sequences rich in adenine and cytosine, particularly at third codon position. The GC3 contents vary within the subgroups, and yet, in general, the subgroups were more similar within than among them. The reasons for the differences in degree of AT-content diversity among subgroups still are unclear, they could be associated with differences in the numbers of representative species among subgroups, differences in the ages of the subgroups, or nucleotide composition evolves faster in some subgroups. Furthermore, the codon usage for the genes COI and COII of the saltans group is similar to the pattern described for insects [57], with high values for the codons TTA (leucine), CGA (arginine), TCT (serine), GGA (glycine). The two clustering methods applied here recovery the saltans and sturtevanti subgroups but fail to recovery the elliptica subgroup due to CUB of COI and COII evolve in a heterogeneous way in D. neosaltans, the earliest to branch off among the elliptica subgroup. More mitochondrial genes should be evaluated to confirm if this is a mitogenome pattern, also evaluation of nuclear genes may be interesting to evaluate the mechanisms that may be shaping the CUB, particularly of D. neosaltans.

In general, the saltans group presents a variety of forms of aedeagus with great complexity, while the external morphology among the species of the group is very similar. This observation can be explained by Eberhard [58], who mentions that the male genitalia of animals subjected to internal fertilization evolve and diverge faster relative to the other morphological characters of the body [58, 59]. Consequently, the great diversity of morphological characters of male terminalia among genetically similar species is related to several complex phenomena driven by selective processes, mainly by sexual selection [58, 60, 61]. Furthermore, it is noteworthy that the rapid evolution of male genitalia is still sufficient to preserve a phylogenetic signal, which is especially useful in comparative and phylogenetic analyses among closely related species [59]. As an example, one study analyzed 41 phylogenetic articles from 11 different orders of arthropods, finding phylogenetically informative characters of the genitalia, suggesting that there is a rapid but ordered evolutionary rate [62]. Likewise, a comparison of 490 characters shows that adult terminalia characters present lower homoplasy than other organs [7].

In the current study, we use the greatest number of male terminalia morphological characters for the saltans group to date, demonstrating robust analysis associated with the most suitable mitochondrial genes for phylogenetic analysis of COI and COII [63, 64]. It is relevant to emphasize the importance of using different markers in phylogenetic analyses as soon as they present different mutation rates and coalescence times, which can provide different information with robust results, often complementary, and increase the accuracy of phylogenetic inferences about the processes involved [3, 4]. Therefore, the combination of these markers
supported the saltans group as monophyletic and a new hypothesis of the relationship among species in the group, such as parasaltans subgroup as a sister taxon of the other species in the saltans group, followed by the formation of two clades: saltans subgroup clustered as sister of the large clade, consisting of the other three subgroups (sturtevanti, cordata and elliptica). Furthermore, the relationship of species within the elliptica and sturtevanti subgroups was well supported.

Supporting information

S1 Fig. Percent contribution of individual codons to the correspondence analysis of RSCU. The contributions of codons to the (A) first and (B) second dimension of correspondence analysis of 16 species of the saltans groups. The red dashed line indicates the expected average value if the contributions were uniform.

S1 Table. Taxonomy and provenance information of the species included in this study.

S2 Table. Primer sequences and PCR annealing temperatures.

S3 Table. RSCU averages of each codon in each species. Bold codons were used in correspondence analysis.

S4 Table. Results of pairwise tests for compositional homogeneity between the 17 studied species for all nucleotides, first, second, and third codon positions. P-values estimated from Monte Carlos test with 1,000 replicates are shown below the diagonal, significant P-values are highlighted.

S5 Table. Base composition test for the saltans species group.

Acknowledgments

We are grateful to Dr. Daniela De Toni for providing some strains from Santa Catarina, Brazil, to Dr. Jean David for the D.septentriosaltans, D.nigrosaltans and MI-2 strains and to Dr. Wolfgang J. Miller for the STV-like strain (D. lehrmanae).

Author Contributions

Conceptualization: Lilian Madi-Ravazzi.

Data curation: Bruna Emilia Roman, Diego J. Santana, Carolina Prediger, Lilian Madi-Ravazzi.

Formal analysis: Bruna Emilia Roman, Diego J. Santana, Carolina Prediger.

Funding acquisition: Lilian Madi-Ravazzi.

Investigation: Bruna Emilia Roman, Lilian Madi-Ravazzi.

Methodology: Bruna Emilia Roman, Carolina Prediger.

Project administration: Lilian Madi-Ravazzi.
Resources: Lilian Madi-Ravazzi.
Software: Carolina Prediger.
Supervision: Lilian Madi-Ravazzi.
Validation: Bruna Emilia Roman, Diego J. Santana, Carolina Prediger, Lilian Madi-Ravazzi.
Visualization: Lilian Madi-Ravazzi.
Writing – original draft: Bruna Emilia Roman, Lilian Madi-Ravazzi.
Writing – review & editing: Bruna Emilia Roman, Diego J. Santana, Carolina Prediger, Lilian Madi-Ravazzi.

References
1. Brandley MC, Schmitz A, Reeder TW. Partitioned Bayesian Analyses, Partition Choice, and the Phylogenetic Relationships of Scincid Lizards. Syst Biol. 2005; 54, 373–390. https://doi.org/10.1080/10635150590946808 PMID: 16012105
2. Levasseur C, Lapointe FJ. War and Peace in Phylogenetics: A Rejoinder on Total Evidence and Consensus. Syst Biol. 2001; 50: 881–891. https://doi.org/10.1080/106351501753462858 PMID: 12116638
3. Templeton AR. Statistical phylogeography: methods of evaluating and minimizing inference errors. Mol Ecol. 2004; 13: 789–809. https://doi.org/10.1046/j.1365-294x.2003.02041.x PMID: 15012756
4. Avise JC. Phylogeography: retrospect and prospect. J Biogeogr. 2009; 36: 3–15.
5. Wiens JJ. The Role of Morphological Data in PhylogenY Reconstruction. Syst Biol. 2004; 53: 653–661. https://doi.org/10.1080/10635150490472959 PMID: 15371253
6. Wiens JJ, Fetzner JW Jr., Parkinson CL, Reeder TW. Hylid Frog Phylogeny and Sampling Strategies for Speciose Clades. Syst Biol. 2005; 54: 778–807. https://doi.org/10.1080/10635150500234625 PMID: 16243760
7. Sayad SA, Yassin A. Quantifying the extent of morphological homoplasy: A phylogenetic analysis of 490 characters in Drosophila. Evol Lett. 2019; 3: 286–298. https://doi.org/10.1002/evl3.115 PMID: 31171984
8. O’Grady PM, DeSalle D. Phylogeny of the genus Drosophila. Genetics. 2018; 209:1–25. https://doi.org/10.1534/genetics.117.300583 PMID: 29716983
9. Throckmorton LH. The phylogeny, ecology, and geography of Drosophila [A]. In: King RC. Handbook of Genetics. Plenum Press. 1975; 3: 421–469.
10. Magalhães LE. Notes on the taxonomy, morphology and distribution of saltans group of Drosophila, with description of four new species. University of Texas Publications. 1962; 6205: 620: 625–154.
11. Mourão CA, Bicudo HEMC. Duas espécies novas de Drosophila do grupo saltans (Drosophilidae, diptera). Pap Avulsos Zool. 1967; 12: 123–134.
12. Guillín ER, Rafael V. Cinco especies nuevas del género Drosophila (Diptera, Drosophilidae) en la provincia de Napo, Ecuador. Iheringia Ser Zool. 2017; 107: e2017022. https://doi.org/10.1590/1678-14766e2017022
13. Santa-Brígida R, Schmitz HJ, Martins MB. Drosophilidae (Insecta, Diptera) in the state of Pará (Brazil). Biota Neotrop. 2017; 17: e20160179. https://doi.org/10.1590/1678-0611-BN-2016-0179
14. Sturtevanti AH. The classification of the genus Drosophila, with descriptions of nine new species. University of Texas Publications. 1942; 421: 5–51.
15. Pavan C, Magalhães LE. The saltans group of Drosophila. In Pavan C (1950) Espécies Brasileiras de Drosophila. Boletim da Faculdade Filosofia, Ciências e Letras, Universidade de São Paulo. 1950; 111: 1–38.
16. Magalhães LE. Description of four new species of the saltans group of Drosophila (Diptera). Rev Bras Biol. 1956; 16: 273–280.
17. Magalhães LE, Björnberg AJ. Estudo da genitalia masculina de Drosophila do grupo saltans (Diptera). Rev Bras Biol. 1957; 17: 435–450.
18. Vilela CR, Bächli G. Taxonomic studies on neotropical species of seven genera of Drosophilidae (Diptera). Mitt Schweiz Ent Ges. 1990; 63: 3–332.
19. Madi-Ravazzi L, Segala LF, Roman BE, Alevi KCC, Prediger C, Yassin A, et al. Integrative taxonomy and a new species description in the sturtevanti subgroup of the Drosophila saltans group (Diptera;
Phylogeny of *Drosophila saltans* group (Diptera: Drosophilidae)

Drosophilidae). Zootaxa. 2021; 4980: 269–292. https://doi.org/10.11646/zootaxa.4980.2.3 PMID: 34186980

20. Bächli G. TaxoDros: The database on Taxonomy of Drosophilidae. 2021. Database: TaxoDros. https://www.taxonodros.uzh.ch/.

21. Bicudo HEMC. Reproductive isolation in the *saltans* group of *Drosophila*. I. The *saltans* subgroup. Genetica. 1973a; 44: 313–329. https://doi.org/10.1007/BF00161311

22. Bicudo HEMC. Chromosomal polymorphism in the *saltans* group of *Drosophila*. I. The *saltans* subgroup. Genetica. 1973b; 44: 520–552. https://doi.org/10.1007/BF00168080

23. Bicudo HEMC. Reproductive isolation in the *saltans* group of *Drosophila*. IV. The sturtevanti subgroup. Rev Bras Genet. 1979; 2: 247–258.

24. O’Grady PM, Clark JB, Kidwel MG. Phylogeny of the *Drosophila saltans* species group based on combined analysis of nuclear and mitochondrial DNA sequences. Mol Biol Evol. 1998; 15: 656–664. https://doi.org/10.1093/oxfordjournals.molbev.a025969 PMID: 9615447

25. Rodriguez-Trelles F, Tarrio R, Ayala FJ. Molecular evolution and phylogeny of the *Drosophila saltans* species group inferred from the Xdh gene. Mol Phylogenet Evol. 1999a; 13: 110–121. https://doi.org/10.1006/mpev.1999.0631 PMID: 10508544

26. O’Grady PM, Clark JB, Kidwel MG. Phylogeny of the subgenus Sophophora (Diptera: Drosophilidae) Based on combined analysis of nuclear and mitochondrial sequences. Mol Phylogenet Evol. 2002; 22: 442–453. https://doi.org/10.1006/mpev.2001.1053 PMID: 11884169

27. Castro JP, Carareto CMA. Elements in the *saltans* group of *Drosophila*: a new evaluation of their distribution and number of genomic insertion sites. Mol Phylogenet Evol. 2003; 32: 383–387. https://doi.org/10.1016/j.ympev.2004.01.005 PMID: 15186822

28. Yassin A. Phylogenetic Relationship Among Species Subgroups in the *Drosophila saltans* Group (Diptera: Drosophilidae): Can Morphology Solve a Molecular Conflict? Zoo Res. 2006; 30: 225–232. https://doi.org/10.3724/SP.J.1141.2009.03225

29. Souza TAJ, Noll FB, Bicudo HEMDC, Madi-Ravazzi L. Scanning electron microscopy of male terminalia and its application to species recognition and phylogenetic reconstruction in the *Drosophila saltans* group. PLoS One. 2014; 9(6): e97156. https://doi.org/10.1371/journal.pone.0097156 PMID: 24915442

30. Rodriguez-Trelles F, Tarrio R, Ayala FJ. Switch in codon bias and increased rates of amino acid substitution in the *Drosophila saltans* species group. Genetics. 1999b; 153: 339–350. https://doi.org/10.1093/genetics/153.1.339 PMID: 10471717

31. Powell JR, Sezzi E, Moriyama EM, Gleason JM, Caccone A. Analysis of a shift in codon usage in *Drosophila*. J Mol Evol. 2003; 57 Suppl 1: S214–25. https://doi.org/10.1007/s00239-003-0030-3 PMID: 15008418

32. Vicario S, Moriyama EN, Powell J.R. Codon usage in twelve species of *Drosophila*. BMC Evol Biol. 2007; 7:226. https://doi.org/10.1186/1471-2148-7-226 PMID: 18005411

33. Wei L, He J, Jia X, Qi Q, Liang Z et al. Analysis of codon usage bias of mitochondrial genome in *Bombyx mori* and its relation to evolution. BMC Evol Biol. 2014; 14: 262. https://doi.org/10.1186/s12862-014-0262-4 PMID: 25515026

34. Kaneshiro KY. A study of the relationships of Hawaiian *Drosophila* species based on external male genitalia. The University of Texas Publication.1969; 6918: 55–70.

35. Roman BE, Madi-Ravazzi L. Male terminalia morphology of sixteen species of the *Drosophila saltans* group Sturtevant (Diptera, Drosophilidae). Zootaxa. 2021; 5061 (3): 523–544. https://doi.org/10.11646/zootaxa.5061.3.7 PMID: 34810610

36. Goloboff PA, Farris JS, Nixon KC. TNT, a free program for phylogenetic analysis. Cladistics. 2008; 24: 774–786. https://doi.org/10.1111/j.1096-0031.2008.00217.x

37. Nixon KC. WinClada. Published by the author. 2002.

38. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 1985; 39: 783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x PMID: 28561359

39. Hall T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999; 44: 95–98.

40. Thompson J, Higgins D, Gibson T. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994; 22: 4673–4680. https://doi.org/10.1093/nar/22.22.4673 PMID: 7984417

41. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 2012; 9: 772. https://doi.org/10.1038/nmeth.2109 PMID: 22847109
64. Palomares-Rius JE, Cantalapiedra-Navarrete C, Castillo P. Cryptic species in plant parasitic nematodes. Nematology. 2014; 16: 1105–1118. https://doi.org/10.1163/15685411-0002831

65. Suvorov A, Kim BY, Wang J, Armstrong EE, Peede D, D’Agostino ERR, et al. Widespread introgression in a malaria vector species complex revealed by phylogenomics. Science. 2015; 347: 1258524. https://doi.org/10.1126/science.1258524 PMID: 25431491

66. Dias GR, Dupim EG, Vanderlinde T, Mello B, Carvalho AB. A phylogenomic study of Steganinae fruit flies (Diptera: Drosophilidae): strong gene tree heterogeneity and evidence for monophyly. BMC Evol Biol. 2020; 20: 141. https://doi.org/10.1186/s12862-020-01703-7 PMID: 33138771

67. Barbhuiya RI, Uddin A, Chakraborty S. Codon usage pattern and its influencing factors for mitochondrial CO genes among different classes of Arthropoda. Mitochondrial DNA Part A. 2020; 31: 313–326. https://doi.org/10.1080/24701394.2020.1800661 PMID: 32755341

68. Eberhard WG. Sexual selection and animal genitalia. Cambridge, MA: Harvard University Press; 1985.

69. Eberhard WG. Evolution of genitalia: theories, evidence, and new directions. Genetica. 2010; 138: 5–18. https://doi.org/10.1007/s10709-009-9358-y PMID: 19308664

70. Arnvist G. The evolution of animal genitalia: distinguishing between hypotheses by single species studies. Biol J Linn Soc Lond. 1997; 60: 365–379. https://doi.org/10.1111/j.1095-8322.1997.tb01501.x

71. Buenaventura E, Pape T. Phylogeny, evolution and male terminalia functionality of Sarcophagidae (Diptera: Sarcophagidae). Zool J Linn Soc. 2018; 183: 808–906. https://doi.org/10.1093/zoolinnean/zlx070

72. Song H, Bucheli SR. Comparison of phylogenetic signal between male genitalia and non-genital characters in insect systematics. Cladistics. 2010; 26: 23–35. https://doi.org/10.1111/j.1096-0031.2009.00273.x PMID: 34875749

73. Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flok P. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann Entomol Soc Am. 1994; 87: 651–701. https://doi.org/10.1093/aesa/87.6.651

74. Ronquist F, Teslenko M, Van der Mark P, Ayres DL, Darling A, Höhna S, et al. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol. 2012; 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029 PMID: 22357727

75. Rambaut A, Drummond AJ. Tracer v1.5. 2007. http://beast.bio.ed.ac.uk/Tracer.

76. Lewis PO. Phylogenetic systematics turns over a new leaf. Trends Ecol. Evol. 2001; 16: 30–37. https://doi.org/10.1016/s0169-5347(00)02025-5 PMID: 11146142

77. Chari D, Lobry JR. SeqinR 1.0–2: A contributed Package to the R Project for statistical computing devoted to biological sequences retrieval and analysis. In: Bastolla U, Porto M, Roman HE, Vendruscolo M. Structural approaches to sequence evolution. Berlin: Springer; 2007.

78. Librado P, Rozas J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 2009; 25: 1451–1452. https://doi.org/10.1093/bioinformatics/btp187 PMID: 19346325

79. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018; 35: 1547–1549. https://doi.org/10.1093/molbev/msy096 PMID: 29722887

80. Wickham H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag. 2016.

81. Lè S, Josse J, Husson F. FactoMineR: An R Package for Multivariate Analysis. J Stat Softw. 2008; 25: 1–18. https://doi.org/10.18637/jss.v025.i01

82. Kopp A, Frank AK, Barmina O. Interspecific divergence, intrachromosomal recombination, and phylogenetic utility of Y-chromosomal genes in Drosophila. Mol Phylogenet Evol. 2006; 38: 731–741. https://doi.org/10.1016/j.ympev.2005.06.016 PMID: 16325432

83. Fontaine MC, Pease JB, Steele A, Waterhouse RM, Neafsey DE, Sharakhov IV et al. Extensive introgression between molecular patterns. Genetics. 2001; 158: 1321–1327. https://doi.org/10.1093/genetics/158.3.1321 PMID: 11454778

84. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018; 35: 1547–1549. https://doi.org/10.1093/molbev/msy096 PMID: 29722887