Title:

Multiple introgressions shape mitochondrial evolutionary history in *Drosophila paulistorum* and the *Drosophila willistoni* group

Running title: mitochondrial evolution in *Drosophila paulistorum*

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ABSTRACT

Hybridization and the consequent introgression of genomic elements is an important source of genetic diversity for biological lineages. This is particularly evident in young clades in which hybrid incompatibilities are still incomplete and mixing between species is more likely to occur. *Drosophila paulistorum,* a representative of the Neotropical *Drosophila willistoni* subgroup, is a classic model of incipient speciation. The species is divided into six semispecies that show varying degrees of pre- and post-mating incompatibility with each other. In the present study, we investigate the mitochondrial evolutionary history of *D. paulistorum* and the willistoni subgroup. For that, we perform phylogenetic and comparative analyses of the complete mitochondrial genomes and draft nuclear assemblies of 25 *Drosophila* lines of the willistoni and saltans species groups. Our results show that the mitochondria of *D. paulistorum* are polyphyletic and form two non-sister clades that we name α and β. Identification and analyses of nuclear mitochondrial insertions further reveal that the willistoni subgroup has an α-like mitochondrial ancestor and indicate that both the α and β mitochondria of *D. paulistorum* were acquired through introgression from unknown fly lineages of the willistoni subgroup. We also uncover multiple mitochondrial introgressions across *D. paulistorum* semispecies and generate novel insight into the evolution of the species.

Keywords: mitochondria, *Drosophila*, evolution, genomics, introgression.

1. INTRODUCTION

Hybridization is increasingly recognized for its importance in promoting genetic diversity as well as for its consequent influence on adaptation and speciation. Large-scale genome sequencing is showing that the process is not only widespread but also relatively common, with many plants, fungi and animals carrying nuclear and cytoplasmic signatures of other species (Edelman and Mallet, 2021; Taylor and Larson, 2019). Genetic elements acquired through introgression — i.e., hybridization followed by back-crossing of hybrids to the parental lineages — often have adaptive value and lead to the emergence of novel traits or increased resistance to environmental stresses (Arnold and Kunte, 2017; Edelman and Mallet, 2021; Oziolor et al., 2019). Once introgressed, genetic elements persist in the receiving species through drift, selection or association with selfish elements that drive their spread (Dumas et al., 2013; Quilodrán, 2020; Seixas et al., 2018). The maternally inherited and reproduction-manipulating symbiont *Wolbachia,* for example, is known to cause sweeps of introgressed mitochondrial types (i.e., mitotypes) across host populations (Dumas et al., 2013; Jiggins, 2003; N Miyata et al., 2020).

The analysis of introgressed genetic elements can contribute to our understanding of past evolutionary events that shaped the present biodiversity (Ottenburghs, 2020). Sequence analysis often allows precise identification of hybridizing lineages and thus generates insight into the evolutionary history and biogeography of organisms of interest (Barlow et al., 2018; Meyer et al., 2012; Taylor and Larson, 2019). However, introgressed elements sometimes do not show strong similarity to any known organisms. “Ghost introgressions” derived from unsampled or potentially extinct species are relatively common and are often detected during the analysis of mitochondrial genomes (Ottenburghs, 2020; Toews and Brelsford, 2012; Zhang et al., 2019). Such introgressions, either from “ghost” or well-known species, commonly lead to deep mitochondrial divergence in clades with closely related nuclear genomes (Hirano et al., 2019; Horoiwa et al., 2021; Makhov et al., 2021; Zadra et al., 2021). If not properly identified, these introgressions can mislead evolutionary analyses (Ottenburghs, 2020; Toews and Brelsford, 2012). Mitochondrial genomic analyses can be further complicated by nuclear mitochondrial insertions (NUMTs) which are found in many organisms. NUMTs sometimes provide important phylogenetic information (Hazkani-Covo, 2009), but they may also create practical problems if mistaken for the true mitochondrial DNA (Calvignac et al., 2011). As in the case of undetected introgressions, unidentified NUMTs sometimes lead to phylogenies that do not represent the true relationship between lineages and to incorrect estimates of biological diversity (DeSalle and Goldstein, 2019; Kress et al., 2015).

Drosophila is a powerful model for studying introgression, as most lineages carry genomic elements acquired from other species or clades (Garrigan et al., 2012; Suvorov et

al., 2022; Turissini and Matute, 2017). In some cases, up to 10% of the genome is estimated to have originated via introgression (Suvorov et al., 2022). Within *Drosophila*, young clades represent particularly interesting study systems, as the lineages which form them are more likely to hybridize or to have done so in the recent past (Coyne and Orr, 1989). One such clade is the *Drosophila willistoni* species group, a recent radiation of Neotropical fruit flies (Zanini et al., 2018) which comprises 24 species split into three subgroups: alagitans, bocainensis, and willistoni. (Bächli, 2019). Among these, the willistoni subgroup is the best studied and comprises several incipient species with varying degrees of inter- and intra-specific reproductive isolation (Burla et al., 1949; Ehrman and Powell, 1982; Winge, 1965). Due to this, the willistoni subgroup has become an important model for investigating reproductive isolation, speciation and hybridization (Burla et al., 1949; Civetta and Gaudreau, 2015; Dobzhansky and Pavlovsky, 1967; Ehrman and Powell, 1982; Mardiros et al., 2016; Perez-Salas and Ehrman, 1971; Schneider et al., 2019; Winge, 1965; Winge and Cordeiro, 1963).

One of the willistoni subgroup species, *Drosophila paulistorum*, is a species complex *in statu nascendi*. It comprises six semispecies — Amazonian (AM), Andean Brazilian (AB), Centro-American (CA), Interior (IN), Orinocan (OR) and Transitional (TR) — that show distinct but partially overlapping geographical distributions (Dobzhansky and Spassky, 1959). The semispecies express variable levels of reproductive isolation and exhibit both pre- and post-mating incompatibilities with each other (Dobzhansky and Spassky, 1959; Ehrman and Powell, 1982). Typically, females discriminate against males of other semispecies and occasional crosses do not produce offspring or result in hybrid infertility and male sterility (Dobzhansky et al., 1964; Ehrman, 1965; Miller et al., 2010). However, the TR semispecies and certain lines of other semispecies are more permissible and sporadically produce viable and, on rare occasions, fertile hybrids, at least under lab conditions (Dobzhansky et al., 1964; Malogolowkin, 1962). Thus, it is possible that gene flow between semispecies still exists or occurred in the recent past, when the semispecies were younger and barriers against gene flow were still incomplete (Perez-Salas et al., 1970). This complex evolutionary scenario has motivated several phylogenetic studies which produced a good understanding of the relationships between species of the willistoni subgroup (Gleason et al., 1998; Robe et al., 2010; Zanini et al., 2018). However, the relationship between *D. paulistorum* semispecies remains somewhat controversial, as nuclear and mitochondrial markers have often produced fairly different phylogenetic trees (Gleason et al., 1998; Robe et al., 2010; Zanini et al., 2018). These incongruences suggest that hybridization and mitochondrial introgression may have occurred between semispecies, but the small number and short sequence length of the markers available in previous studies have not allowed definite conclusions to be reached.

94 In the present study, we investigate the evolutionary history of mitochondria in *D.*
95 *paulistorum.* We use complete mitochondrial genomes and draft whole-genome assemblies
96 of 25 *Drosophila* lines belonging to six species of the willistoni group and three species of
97 the closely related saltans group. By comparing mitochondrial and nuclear phylogenies we
98 uncover a complex evolutionary history involving multiple mitochondrial introgressions, both
99 ancient and recent. We discover that *D. paulistorum* mitochondria are polyphyletic and form
100 two distinct non-sister clades, which we name α and β. With our analyses of the α and β
101 genomes as well as of NUMTs found in the *D. paulistorum* genome, we show that the
102 willistoni subgroup has an α-like mitochondrial ancestor. Interestingly, we also find evidence
103 that both current *D. paulistorum* mitotypes are not native to the species and were likely
104 acquired through introgression. The current α mitotype appears to have introgressed prior to
105 the divergence of *D. paulistorum* and *D. equinoxialis.* As for the β mitotype, we suggest that
106 it was acquired by *D. paulistorum* prior to semispecies diversification through a ghost
107 introgression from an unsampled or extinct lineage of the willistoni group. Our results further
108 reveal multiple introgressions across semispecies of *D. paulistorum*, generating new insight
109 into the evolution of the species.

110 2. MATERIAL AND METHODS

111 2.1. Fly lines, DNA extraction and sequencing

112 *Drosophila* lines used in this study are summarized in **Table 1**. Flies were kept at 25±1°C on
113 Formula 4-24 *Drosophila* instant food (Carolina, USA) with a 12-hour light-dark cycle. For
114 each line, 20 ovaries were dissected from 3-day-old adult females and DNA extraction was
115 performed using the Gentra Puregene Blood and Tissue kit (Qiagen). DNA samples used for
116 PacBio sequencing were produced using the protocol described in (Ellegaard et al., 2013).
117 Briefly, *Drosophila* flies were transferred to apple juice agar plates and allowed to oviposit for
118 2 hours, after which the eggs were collected, washed and dechorionated in 50% bleach and
119 manually homogenized using a plastic pestle. Following centrifugation and filtration of the
120 homogenate, the resulting cell pellet was subjected to whole genome amplification using the
121 Repli-g midi kit (Qiagen) after which the DNA was purified using QIAamp DNA mini kit
122 (Qiagen) according to the manufacturer's recommendations.

123 *2.1.1. Sequencing*

124 *Drosophila paulistorum* lines from the six classic reference semispecies plus lines from more
125 recent collections were used for genome sequencing (**Table 1)**. DNA extracted from ovaries
126 was used to construct 350 bp fragment TruSeq libraries, multiplexed and sequenced at the
127 Uppsala SNP and Seq platform in three different runs on an Illumina 2500 HiSeq machine
128 generating 2x125 bp reads. The Illumina reads were quality filtered and trimmed using

129 Trimmomatic-0.30 (Bolger et al., 2014) and error-corrected using SPAdes-3.5.0 (Bankevich
130 et al., 2012). Amplified DNA extracted from early embryos of three lines (O11, POA-1 and
131 TP37) was used to create 5 kb fragment SMRTbell libraries. Each library was run using P6-
132 C4 chemistry in one SMRT cell on an RSII PacBio instrument. PacBio libraries were
133 produced and sequencing was performed at the Uppsala Genome Center, Uppsala,
134 Sweden.

135 **2.2. Genome assemblies and annotation**

136 *2.2.1. Mitochondrial genome assembly*

137 The mitochondrial genome of the O11 line was generated by mapping all Illumina reads to
138 the published mitochondrial genome of *D. willistoni* (NCBI accession number BK006338.1)
139 using BWA-MEM v0.7.17 (Li, 2013) and thereafter extracting all aligned reads and their
140 mates using SAMtools v1.14 (Li et al., 2009). The extracted Illumina reads were then
141 subsampled to achieve an approximate coverage of 100 and assembled together with
142 PacBio filtered subreads using SPAdes-3.10.1 with various k-mers settings. The genome
143 came out as a single full-length contig. Both the Illumina and PacBio reads were then
144 aligned to this contig using BWA-MEM v0.7.17. The resulting bam-file was converted to an
145 ace-file that was used for manual curation of the sequence in Consed (Gordon et al., 1998).
146 The mitochondrial genomes of all other *Drosophila* lines were generated by mapping
147 Illumina reads to the O11 mitochondrial genome with BWA-MEM v0.7.17, extracting all
148 aligned reads and their mates using SAMtools v1.14 and assembling the extracted reads
149 with SPAdes-3.10.1 with various k-mers settings. Since PacBio data were only generated
150 from the O11, TP37 and POA-1 lines, the assemblies of all other lines were run using only
151 Illumina reads. For all lines except FG572, this procedure generated one contig that
152 represented a complete or nearly complete circular mitochondrial genome. For FG572, this
153 procedure created two nearly equal length contigs with similar coverage. The longest contig
154 of ca. 14 kbp was similar to other β mt-genomes. The other large contig of ca 13 kbp was
155 similar to α mt-genomes, and specifically to the NUMT that was discovered in the strain L06
156 (described in 3.3). Hence, this contig was regarded as a NUMT and not a real mitochondrion
157 (see 2.6). To complete the mt-genome of FG572, we joined the 14 kbp β contig to a shorter
158 contig of ca. 2000 bp which had almost double coverage and which represented the missing
159 part of the mt-genome. The double coverage was due to the high similarity between the α-
160 like NUMTand β mt-genome in this region.

161 In order to correct possible assembly errors, Illumina reads of each line were
162 mapped back to the contig containing the complete or nearly complete mt-genome for that
163 line using BWA-MEM v0.7.17 and indels were realigned with the IndelRealigner from GATK
164 (McKenna et al., 2010). The resulting bam-file was then used to call variants using

165 Freebayes with a range of parameters and converted to ace-format for manual curation in
166 Consed (Gordon et al., 1998). In cases where high-frequency variants existed, we used the
167 majority rule and base quality to select the consensus base.
168 The position and function of genes in the assembled mt-genomes were obtained by
169 extracting the gene sequences from the published *D. willistoni* mt-genome (GD-H4-1 line)
170 and using them as queries in a blastn search against each of our assembled mt-genomes.
171 The start and stop positions of each gene were subsequently checked manually in Artemis
172 (Rutherford et al., 2000) and adjusted if needed.

173 *2.2.2. Nuclear genome assemblies and retrieval of nuclear markers*
174 Illumina reads from each *Drosophila* line were separately assembled with Megahit v.1.1.2 (Li
175 et al., 2015) using multiple k-mer settings to generate draft nuclear genome assemblies.
176 BUSCO v5.2.2 (Manni et al., 2021) was used for estimating the completeness of each
177 assembly based on the 'Diptera' marker dataset. Complete single copy BUSCO markers
178 which could be retrieved from all assemblies (**Table S3**) were then used for building nuclear
179 phylogenies, as described in 2.3.

180 **2.3. Alignments and phylogenetic analyses**
181 Datasets of whole mitochondrial genomes, nuclear markers and mitochondrial genes were
182 aligned with MAFFT v7.490 (mafft-linsi algorithm) (Katoh and Standley, 2013) and
183 processed with TrimAl v1.4rev15 (Capella-Gutierrez et al., 2009) using the -automated1 flag
184 for trimming poorly aligned regions. Concatenated sets of either mitochondrial or nuclear
185 genes were created by combining trimmed alignments of single genes. Datasets of
186 mitochondrial protein-coding genes containing either only the 3rd or a combination of 1st and
187 2nd codon positions were created by parsing gene sequences using an in-house script.
188 Alignments were visually inspected with Aliview v. 2019 (Larsson, 2014) for quality control.
189 Maximum likelihood trees were built with RAxML v8.2.12 (Stamatakis, 2014) using
190 the flags: -f a -m GTRGAMMA -x 12345 -p 12345 -# 1000 (nucleotide trees) or: -f a -m
191 PROTGAMMAGTR -x 12345 -p 12345 -# 1000 (amino acid trees). Bayesian trees were built
192 with MrBayes v3.2.7a (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003)
193 using "nst= mixed" for estimating evolutionary models. Analyses were run for 10.000.000
194 generations with "nruns= 2", "nchains= 4" and "samplefreq= 1000". A burn-in of 25% was
195 applied. Trees were visualized in FigTree v. 1.4.3 (Rambaut, 2009) and rooted using the
196 species of the *saltans* group.

197 **2.4. Quantification of synonymous and non-synonymous substitutions**
198 In order to quantify synonymous and non-synonymous substitutions between the α and β
199 clades, we first generated single gene alignments for all mitochondrial protein-coding genes

200 using MAFFT v7.490. Synonymous and non-synonymous substitutions were counted in all
201 possible pairwise comparisons between α– and β-carrying lines using codeml (PAML
202 package v. 4.9j) (Yang, 2007). The values obtained were then averaged for each mitotype
203 and gene. The same method was used for calculating the number of nonsynonymous
204 substitutions per non-synonymous site (dN), synonymous substitutions per synonymous site
205 (dS) and dN/dS ratios.

206 **2.5. Recombination analysis**

207 We checked for intergenic recombination between lines by analyzing incongruences
208 between single gene trees which were built as described in 2.3. Tests for intragenic
209 recombination on single-gene and whole-genome alignments were performed with
210 Geneconv v.1.81 and PhiPack using default parameters.

211 **2.6. Identification of nuclear mitochondrial DNA (NUMTs)**

212 Using blastn, we screened our draft genome assemblies of willistoni subgroup lines for
213 contigs which produced high-scoring pairs (HSPs) of at least 200 bp and 90% similarity to
214 any of the assembled *D. paulistorum* mt-genomes. Among these, contigs with HSPs with
215 98% or higher identity to either α or β mt-genomes were tentatively assigned to those
216 clades, while contigs with HSPs with 90-97% identity were deemed divergent. We further
217 analyzed their similarity to the α and β mitotypes and calculated their coverage by using
218 BWA-MEM v0.7.17 to map the Illumina reads of each line to their respective whole-genome
219 assemblies combined with a representative of the α (C2) and β (O11) mitotypes. Read
220 coverage was calculated with Mosdepth v0.3.2 (Pedersen and Quinlan, 2018) taking all
221 mapped reads into account. Contigs with coverage lower than two were removed from the
222 analysis to avoid potential artifacts. Contigs with similarity to mitochondria were considered
223 NUMTs if their average coverage was similar (± 30%) to the nuclear coverage of their line.
224 Additionally, contigs were classified as NUMTs if part of their sequence had similarity to the
225 *Drosophila* nuclear genome, as indicated by blast searches against the NCBI nt database.
226 The nuclear coverage of each line was defined as the average coverage of the 10 longest
227 BUSCO markers used in our nuclear phylogenetic analysis (**Table S3**). We also calculated
228 the percentage of bases of the α and β reference genomes that were covered by mapped
229 reads using SAMtools mpileup. The result of these analyses is summarized in **Table S4**.

230 3. RESULTS

231 To investigate the evolutionary history of mitochondria in *D. paulistorum*, we sequenced and
232 assembled the complete mitochondrial (mt) genomes and draft nuclear genomes of 23
233 *Drosophila* lines representing six species of the willistoni group and three species of the

234 closely related saltans group (**Table 1**). Among these were 13 lines of *D. paulistorum*,
235 including representatives of the six classic semispecies and seven recently collected
236 isofemale lines from Brazil and French Guiana (**Table 1**). Additionally, we assembled the mt-
237 genome of the *D. paulistorum* line L06/ H66.1C (**Table 1**), for which whole-genome Illumina
238 data is publicly available (Kim et al., 2021).

Table 1. Fly lines used in this study

Species (semispecies)	Line	Collection locality*	Source/Collector	Collection date
D. paulistorum (AB)	MS	Mesitas, CO	L.E.	1962
D. paulistorum (AM)	A28	Belém (PA), BR	L.E.	1952
D. paulistorum (CA)	C2	Lancetilla, HN	L.E.	1954
D. paulistorum (IN)	L1	Llanos, CO	L.E.	1958
D. paulistorum (OR)	O11	Georgetown, GY	L.E.	1957
D. paulistorum (TR)	SM	Santa Marta, CO	L.E.	1956
D. paulistorum	FG572	Saül, GF	W.M. & A.H.	2015
D. paulistorum	FG103	Saül, GF	W.M. & A.H.	2014
D. paulistorum	FG111	Saül, GF	W.M. & A.H.	2014
D. paulistorum	FG295	Saül, GF	W.M. & A.H.	2014
D. paulistorum	L06/ H66.1C	San Salvador, SV	W.H. [1]	1955
D. paulistorum	POA1	Porto Alegre (RS), BR	A.G.	2003
D. paulistorum	RP	Ribeirão Preto (SP), BR	A.G.	1995
D. paulistorum	TP37	Pousada Triunfo (PE), BR	C.R.& A.G.	2009
D. equinoxialis	FS	Fort Sherman, Colon, PA	E.B.	2002
D. insularis	MASS-B-SL	LC	J.P.	2006
D. tropicalis	SS	San Salvador, SV	W.H.[2]	1955
D. willistoni	APA8-2	Veracruz, ME	J.S.	1998
D. willistoni	FG168	Saül, GF	W.M. & A.H.	2014
D. willistoni	GD-H4-1	GP	J.P.[3]	1991
D. nebulosa	NEB	Palmira, CO	W.H.[4]	1955
D. neocordata	NEO	Minas Gerais, BR	NDSSC[5]	1959
D. prosaltans	PE-2	Eldorado (RS), BR	J.S.	1995
D. prosaltans	Tap5-2	S.Lour. da Mata (PE), BR	C.R.& A.G.	2011
D. septentriosaltans	PLR	Gamboa, PA	E.B.	2002

239 *Abbreviations between brackets refer to Brazilian states.
240 Abbreviations: Collectors: A.G.: Ana Laurer Garcia; AH: Aurélie Hua-Van; C.R.: Claudia Rohde; E.B: Egon Bartel; J.P.: Jeff
241 Powell; J.S.: Joana Silva; L.E.: Lee Ehrman; W.H: William Heed; W.M.: Wolfgang Miller. Countries: BR: Brazil; CO: Colombia,
242 HN: Honduras, GF: French Guiana, GP: Guadeloupe, GY: Guyana, LC: St Lucia, ME: Mexico, SV: El Salvador, PA: Panama.
243 Brazilian states: PA: Pará, PE: Pernambuco, RS: Rio Grande do Sul, SP: São Paulo. Other: NDSSC: National *Drosophila*
244 species stock center. NDSSC codes: [1:]14030-0771.06, [2:]14030-0801.01, [3:]14030-0811.24, [4:]14030-0761.00, [5:]14041-0831.00

245 The assembled mt-genomes are approximately 16.000 bp long, have a GC content of
246 roughly 21% (**Table S1**) and encode the same 13 proteins, 22 tRNAs and two rRNAs as the
247 mitochondria of *D. melanogaster* and most other animals. All mt-genomes have relatively
248 short control regions (CR) of approximately 1.1 kb (willistoni group) to 1.3 kb (saltans group)
249 compared to the 4.6 kb CR of *D. melanogaster*.

The draft nuclear assemblies have an average total length of 177 Mbp and vary considerably in the number of contigs, total length and quality (**Table S1**).

3.1. The phylogeny of *Drosophila paulistorum* and the willistoni group

We inferred a mitochondrial phylogeny of our fly lines based on our newly assembled mt-genomes and the mt-genome of the published *D. willistoni* reference genome GD-H4-1. The resulting tree revealed that *D. paulistorum* mitochondria are polyphyletic and split into two major clades, which we designate α and β (**Fig 1A**). The mt-genomes of the two clades are relatively distantly related and have an average nucleotide divergence of 2.4%. An analysis of substitution patterns between α and β showed that the rate of synonymous substitutions (dS) within protein-coding genes is higher than that of non-synonymous substitutions (dN) (**Table S2**), which indicates that purifying selection is acting on both clades.

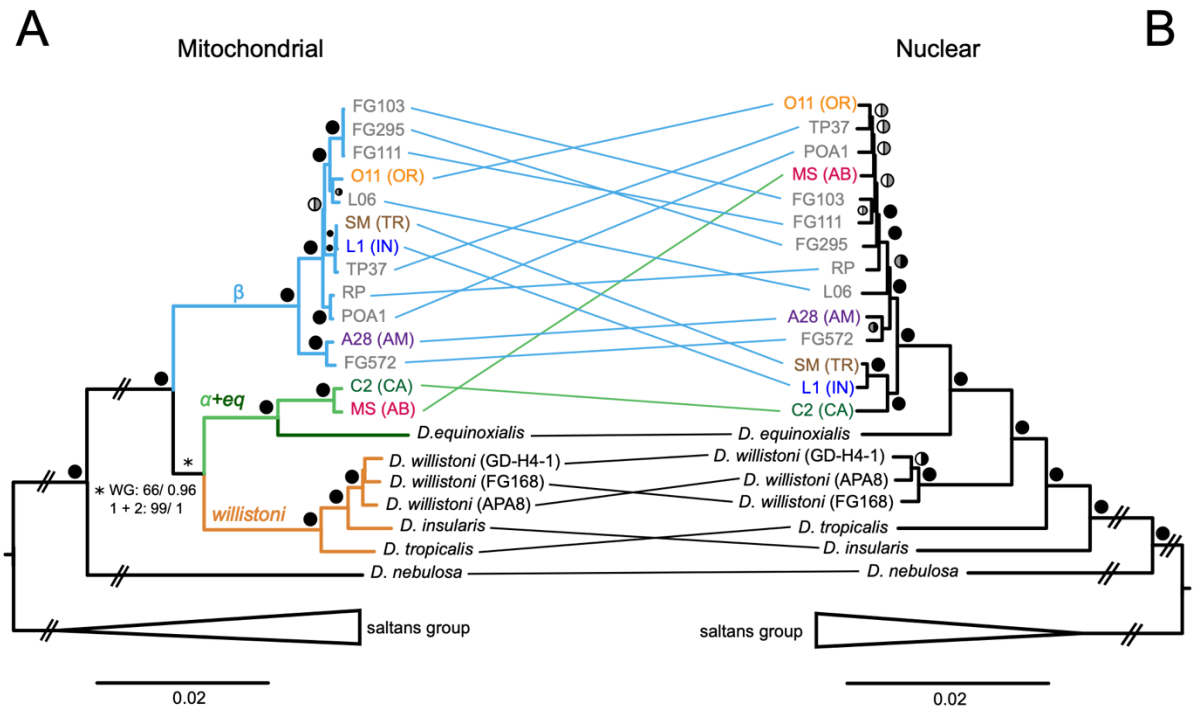
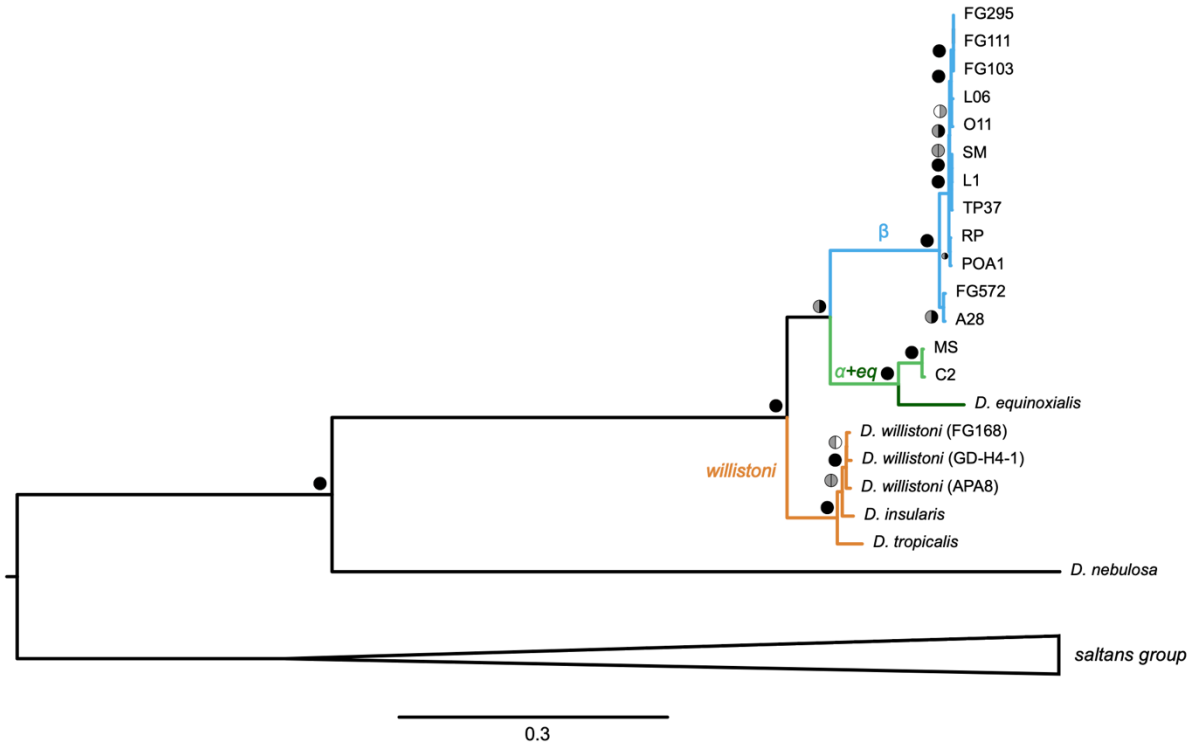


Fig 1. Phylogeny of *D. paulistorum* and the willistoni group based on **(A)** whole mitochondrial genomes and **(B)** nuclear markers. *D. paulistorum* mitochondria form two non-sister clades: α and β. In the mitochondrial tree, the clade containing α and the mitochondria of *D. equinoxialis* (α+eq) is sister to the other species of the willistoni subgroup (willistoni clade). Support for this relationship is low in the whole genome (WG) phylogeny but high when amino acids or only the first and second codon positions of protein-coding genes are analyzed (1+2). The nuclear phylogeny shows *D. paulistorum* semispecies split into two sister clades with three semispecies each. For both trees, the same topology is obtained by maximum likelihood (ML, GTRGAMMA model) and Bayesian analyses. Colored circles next to the nodes indicate ML bootstrap support (left half) and Bayesian posterior probability (right half). Black represents 99-100% support, grey 70-98%, and white below 70%. Branches marked by transverse lines were shortened and do not follow the scale bar.

We found all deep nodes of the mitochondrial tree to be highly supported, except one (**Fig 1A**). The unsupported node is essential for our understanding of the phylogenetic relationships between *D. paulistorum* mitochondria since it defines the relationship between the clade which contains both the *D. paulistorum* α mitotype and the *D. equinoxialis* mt-

276 genome (hereafter called "α+eq") in regard to the clade containing the mt-genomes of *D.*
277 *willistoni*, *D. insularis* and *D. tropicalis* (hereafter called the "willistoni clade") (**Fig 1A**). Since
278 further conclusions about the evolution of *D. paulistorum* mitochondria can only be made
279 based on a robust phylogeny, we investigated possible underlying reasons for the low
280 support of this node.
281 We tested if recombination between mitotypes could be affecting the phylogenetic
282 signal by generating single gene trees for each of the 13 mitochondrial protein-coding genes
283 and looking for conflicts between their topologies. We found that most of the trees had poor
284 resolution due to the short sequence length of individual genes and low divergence between
285 lines. Only two gene trees (ND2 and ND5) had good support for the node defining the
286 relationship between α+eq and the willistoni clade (Bootstrap values > 80) (**Fig S1**). The
287 ND2 tree featured α+eq as sister to the willistoni clade (BS = 80%) (**Fig S1A**) while the ND5
288 tree placed α+eq as sister to the β clade (BS = 92%) (**Fig S1B**). Among all single gene
289 trees, *ND5* was the only one with support on all internal nodes, perhaps because it is the
290 longest mitochondrial gene. We further tested for intragenic recombination in all protein-
291 coding genes using two different algorithms and found that only *cytB* had a marginally
292 significant signal ($p<0.05$) with one method, Phipack (see Methods). These weak and
293 inconsistent signals indicated that recombination between mt-genomes is probably
294 infrequent and thus unlikely to be the cause of the low bootstrap support in the tree.
295 Next, we tested if nucleotide composition bias could be responsible for the low
296 bootstrap support. For that we built trees based on the concatenated sequences of the 13
297 mitochondrial protein-coding genes using either 1) amino acid (aa) sequences, 2) only the
298 first and second codon positions or 3) only the third codon position. Support for α+eq as
299 sister to the willistoni clade was strongly increased in the tree based on aa sequences (ML
300 bootstrap 90%) in comparison to the whole mt-genome phylogeny (ML bootstrap 66%) (**Fig**
301 **1A, Fig S2A**). Using the first two codon positions of the nucleotide sequences of the protein-
302 coding genes also resulted in considerably higher support for α+eq as sister to the willistoni
303 clade (ML 99%, Bayesian 1) (**Fig 1A, Fig S2B**). In contrast, the tree based only on the third
304 codon position showed high (Bayesian posterior probability 0.99) or fairly good (ML
305 bootstrap 90%) support for α+eq and β being sister clades (**Fig 2**). Thus, given the
306 conflicting topologies, we conclude that nucleotide composition bias is likely causing the low
307 support of the node defining the relationship between the α+eq and willistoni clades in the
308 mitochondrial phylogeny.
309 Since the third codon position is more prone to bias than the first and second
310 positions, we considered the topology supported by amino acids and by the first two codon
311 positions to most likely represent the accurate evolutionary relationship between the clades.
312 This is the same topology seen in the whole mt-genome tree (**Fig 1A**). Thus, we confirmed

313 that the mitochondria of *D. paulistorum* are polyphyletic and that α+eq is sister to the
314 willistoni clade rather than to β **(Fig 1A)**. The result also indicates that the ancestral
315 mitochondrion of the willistoni subgroup is more similar to α (thus "α-like") than to β.



316 **Fig 2. Mitochondrial phylogeny of *D. paulistorum* and the willistoni group based on the third**
317 **codon position of the concatenated protein-coding gene sequences**. The α+eq clade is highly
318 supported as sister to β, which contrasts with its position as sister to the willistoni clade in trees based
319 on whole mitochondrial genomes and on the first and second codon positions of the concatenated
320 mitochondrial protein-coding genes (**Fig 1A, Fig S2B**). The change in topology is likely a result of
321 convergence in nucleotide composition due to the ancestor of α+eq and β having lived in the same
322 host. The same topology is observed in maximum likelihood (ML, GTRGAMMA model) and Bayesian
323 analyses. Colored circles next to the nodes indicate ML bootstrap support (left half) and Bayesian
324 posterior probability (right half). Black represents 99-100% support, grey 70-98%, and white below
325 70%.

326 To investigate the evolution of *D. paulistorum* mitochondria in more detail, we also inferred a
327 reliable nuclear phylogeny that reflects the evolutionary history of the species and its
328 semispecies (**Fig 1B**). The tree was based on amino acid sequences of 692 BUSCO
329 markers ('Diptera' dataset) which were recovered as complete single copies in all of our draft
330 nuclear assemblies (**Table S3**). The resulting nuclear phylogeny is highly supported in most
331 non-terminal nodes and shows that the six classical semispecies of *D. paulistorum* are split
332 into two sister clades, one formed by the AM, OR and AB semispecies and the other by the
333 CA, IN and TR semispecies (**Fig 1B**).

334 **3.2. Mitochondrial evolutionary history of *D. paulistorum* and the willistoni**
335 **subgroup**

336 A comparison of our nuclear and mitochondrial phylogenies (**Fig 1**) revealed inconsistencies
337 that suggest both ancient and recent mitochondrial introgressions in *D. paulistorum*. First,
338 the phylogenetic position of the β clade indicates that this mitotype was likely acquired by *D.*
339 *paulistorum* from a donor species that diverged earlier than the willistoni subgroup but later
340 than *D. nebulosa*. Second, the placement of the mitochondrial α+eq clade as sister to the
341 willistoni clade is in strong contrast to the derived position that *D. paulistorum* and *D.*
342 *equinoxialis* occupy within the willistoni subgroup in the nuclear phylogeny (**Fig 1**). This
343 indicates that both extant mitotypes of *D. paulistorum* were potentially introgressed from
344 species that branch outside of the willistoni subgroup.

345 Third, more recent mitochondrial introgressions appear to have occurred between
346 the semispecies of *D. paulistorum.* This is evident by the fact that lines from different and
347 relatively distantly related semispecies (based on our nuclear phylogeny) share the same
348 mitotype, as is the case of the α mitotype in C2 (CA) and MS (AB) and the β mitotype in O11
349 (OR), L1 (IN) and SM (TR) (**Fig 1A**).

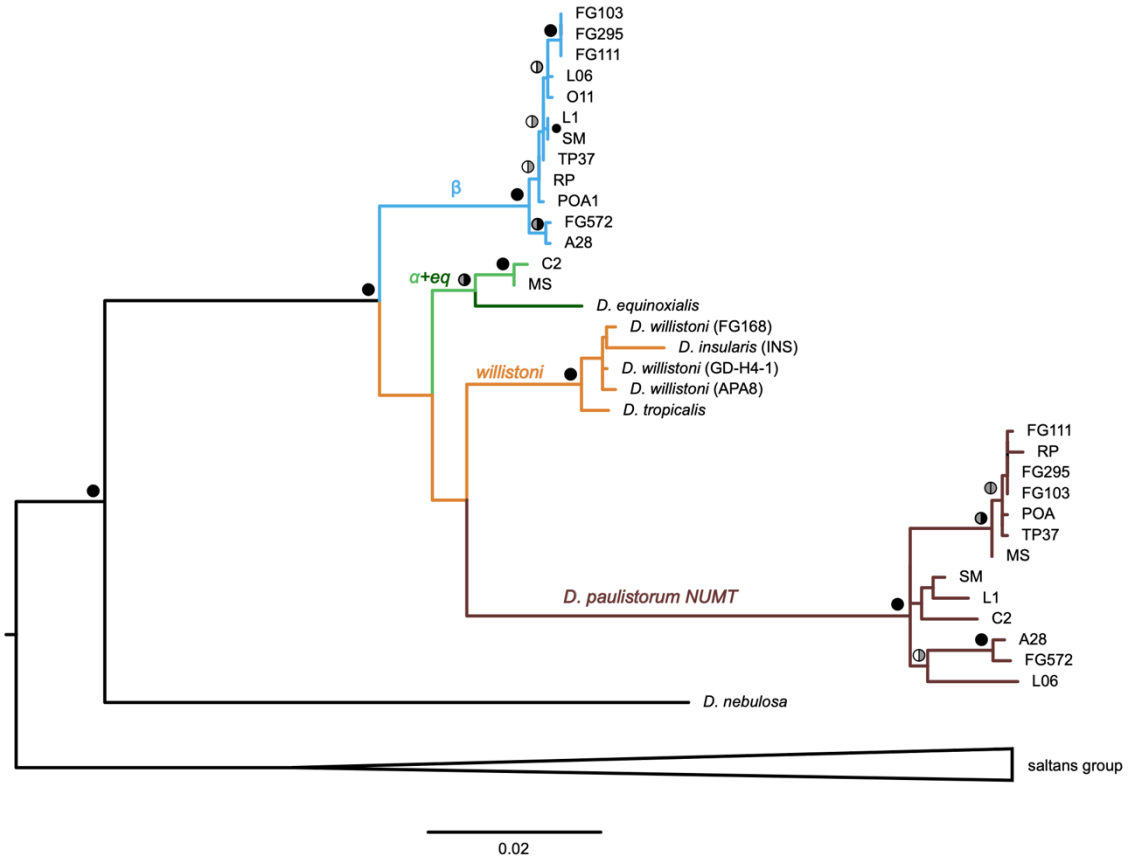
350 Finally, we note that *D. tropicalis* and *D. insularis* switch positions between the
351 nuclear and mitochondrial phylogenies (**Fig 1**).

352 **3.3. NUMTs provide further insight into mitochondrial evolution of the willistoni**
353 **subgroup**

354 While assembling the *D. paulistorum* genomes, we discovered that several of our lines likely
355 carry nuclear mitochondrial insertions (NUMTs). We analyzed these to verify the accuracy of
356 our assembled mt-genomes and to better understand mitochondrial evolution within the
357 willistoni subgroup. Using our whole-genome assemblies, we identified contigs containing
358 sequence fragments that showed at least 90% sequence similarity to representatives of the
359 α and the β mitotypes of *D. paulistorum.* We then investigated their coverage and further
360 analyzed their similarity to α and β by mapping reads of each line to their respective whole-
361 genome assemblies plus representatives of α and β mt-genomes (see 2.6, **Table S4**).
362 Contigs were classified as NUMTs if they either had a coverage similar to that of known
363 nuclear genes or if they contained sequences identified as being of nuclear origin,
364 regardless of coverage. The identified potential NUMTs exhibited varying lengths and
365 degrees of sequence similarity to the assembled mt-genomes. Three of them were of
366 particular interest for interpretations regarding mitochondrial evolution in *D. paulistorum*.

367 First, in several of the *D. paulistorum* lines, we identified a NUMT that lacks many of
368 the typical hallmarks of a nuclear mitochondrial insertion. It spans the whole length of a
369 mitochondrial genome (**Table S4**) and is almost identical to the α mitotype (99,4%

370 nucleotide identity to a consensus of the C2 and MS mt-genomes). This atypical NUMT
371 initially led us to believe that the lines which carry it were heteroplasmic. However, its
372 identity as a NUMT was established due to a clear association with nuclear sequences in the
373 published genome assembly of the *D. paulistorum* L06 line (Kim et al., 2021) as well as in
374 contigs found in our draft whole-genome assemblies of the FG103, FG111, IN, O11 and SM
375 lines. The high similarity between this NUMT and the α mitotype indicates that it is a recent
376 nuclear transfer. However, most lines that carry it have a β mt-genome, suggesting that this
377 NUMT does not originate from recent nuclear insertions of the resident mt-genomes. Thus, it
378 must have been transferred between lines and semispecies in sporadic hybridization events.
379 We are currently investigating the genomic and biological properties of this NUMT and
380 intend to publish our results in a future publication.
381 The second NUMT of interest spans a genomic region that includes parts of the *ND2*
382 and *CO1* genes. This NUMT was found in 13 of the 14 analyzed *D. paulistorum* lines and
383 using it for phylogenetic analysis revealed a relationship between lines that is similar to that
384 seen in the nuclear phylogeny (**Fig 3**, **Fig 1B**).



385 **Fig 3. NUMT-derived phylogenetic relationship between *D. paulistorum* lines.** The phylogeny is
386 based on a NUMT sequence from *D. paulistorum* which covers part of the *ND2-CO1* genes combined
387 with fragments of the true mt-genomes spanning the same region. The recovered phylogenetic
388 relationship between *D. paulistorum* lines resembles that seen in the nuclear marker tree (**Fig 2B**),
389 supporting an ancient origin of this nuclear insertion. The position of the NUMT next to the willistoni
390 and the α+eq clades supports an α–like mitochondrial ancestor in the willistoni subgroup. Colored
391 circles next to the nodes indicate ML bootstrap support (left half) and Bayesian posterior probability
392 (right half). Black represents 99-100% support, grey 70-98%, and white below 70%.

393 This observation, combined with the presence of the NUMT in nearly all *D. paulistorum* lines,
394 suggests that it stems from a single nuclear insertion that occurred before the diversification
395 of *D. paulistorum* semispecies. Additionally, we noticed that the C2, L1 and SM lines share a
396 unique insertion of a CR-1 family retrotransposon in this NUMT, further indicating that it is
397 likely an old nuclear insertion. The presence of this retrotransposon also supports the
398 monophyletic clade formed by the CA, IN and TR semispecies in our nuclear phylogeny (**Fig**
399 **1B**). The ancient origin of this NUMT combined with its phylogenetic position close to α and
400 the willistoni clade (**Fig 3)** corroborates the hypothesis of an α-like ancestral mitotype in *D.*
401 *paulistorum*.
402 Finally, we observed that the genome assembly of *D. equinoxialis* contains a few β-
403 mitotype-related contigs which were not found in *D. insularis*, *D. tropicalis* or *D. willistoni.*
404 The coverage of these contigs is similar to the nuclear average for the line but they do not
405 contain matches to non-mitochondrial sequences. By investigating the coverage of these
406 contigs, we saw that β-related reads present in our *D. equinoxialis* sample cover the whole
407 extent of a mt-genome and result in a full mt-genome when assembled (**Table S4**). Based
408 on the available data, we cannot be sure if these sequences stem from NUMTs or if they
409 represent low-level contamination. Nevertheless, it is interesting to note that the presence of
410 β sequences in *D. equinoxialis* might suggest, if true, that the last common ancestor of *D.*
411 *paulistorum* and *D. equinoxialis* carried a β-like mitochondrion.

412 4. DISCUSSION

413 In this study, we investigate the evolutionary history of *D. paulistorum* using the complete
414 mitochondrial genomes and draft nuclear assemblies of 25 *Drosophila* lines of the willistoni
415 and saltans groups, including 14 *D. paulistorum* lines. We discover that *D. paulistorum*
416 mitochondria are polyphyletic, forming two non-sister clades which we name α and β. We
417 determine that the willistoni subgroup had an α-like mitochondrial ancestor and uncover
418 multiple mitochondrial introgressions between *D. paulistorum* semispecies as well as across
419 *D. paulistorum* and other unidentified lineages of the willistoni group. These corroborate
420 previous findings of frequent hybridization in *Drosophila* (Suvorov et al., 2022) and add to
421 reports of mitochondrial introgression across closely related *Drosophila*, as seen in the *D.*
422 *yakuba* and *D. simulans* groups (Garrigan et al., 2012; Turissini and Matute, 2017). Using
423 phylogenomics, we recover a nuclear phylogeny of *D. paulistorum* which is largely
424 consistent with previous findings but does not recover either the AM or the TR semispecies
425 as early-diverging and ancestral to the other semispecies, as proposed in other studies
426 (Chao et al., 2010; Spassky et al., 1971; Zanini et al., 2018). Instead, we recover *D.*
427 *paulistorum* semispecies split into two sister clades of three semispecies each. Our data

428 also indicate that *D. paulistorum* carries several NUMTs, as previously seen in other
429 *Drosophila* species including the relatively closely related *D. willistoni* (Rogers and Griffiths-
430 Jones, 2012).

431 **4.1. Evolutionary history of the α and β mitotypes of *D. paulistorum***

432 Our analysis shows *D. paulistorum* mitochondria to be polyphyletic and to form two non-
433 sister clades that we name α and β. Both mitotypes show an early-diverging phylogenetic
434 position which contrasts with the derived position of *D. paulistorum* within the willistoni
435 subgroup (Gleason et al., 1998; Robe et al., 2010; Zanini et al., 2018). Thus, if our tree
436 represents the true evolutionary history of these mitochondria, we can conclude that α and β
437 are not originally from *D. paulistorum* but instead were introgressed from unknown early-
438 diverging lineages. The same is true for the mitochondrion of *D. equinoxialis,* which is sister
439 to the α mitotype (forming the α+eq clade) but branches outside *D. paulistorum* in the
440 nuclear phylogeny. The positions of α+eq and of β in the mitochondrial tree suggest that
441 both clades originate from lineages phylogenetically positioned between *D. nebulosa* and
442 the willistoni subgroup, with the donor of β branching out earlier. We note that none of the
443 sampled lineages of the willistoni group (except for *D. paulistorum*) has β-like mitochondria
444 and that a search in GenBank reveals that α is the closest known relative to β. Hence, the
445 donor lineage of β must be either still unsampled or extinct. One possibility is that α and β
446 originate from rare and unsampled lineages in the alagitans or bocainensis subgroups of the
447 willistoni group (Wheeler and Magalhães, 1962; Zanini et al., 2015). Most species in these
448 subgroups are poorly known and have never been sequenced. Thus, it is possible that some
449 occupy the likely phylogenetic position of the potential donors of α and β, i.e., independent
450 lineages which are closer to the willistoni subgroup than to *D. nebulosa*. Ghost
451 introgressions from unknown or extinct donors are reported in several organisms and often
452 result in deep mitochondrial divergence among lineages with high nuclear similarity
453 (Ottenburghs, 2020; Zhang et al., 2019), a situation comparable to what we observe in *D.*
454 *paulistorum* and the willistoni subgroup.

455 Our analyses also allow us to infer at which point of the evolution of the willistoni
456 subgroup the introgressions of α and β took place. Given the phylogenetic position of α as
457 sister to the mitochondrion of *D. equinoxialis*, we conclude that the common ancestor of
458 α+eq was introgressed into the common ancestor of *D. paulistorum* and *D. equinoxialis*. The
459 potential β-NUMTs found in the genome of *D. equinoxialis* suggest that the same could be
460 true for β. However, since we could not conclude if these β-like sequences in *D. equinoxialis*
461 are indeed NUMTs or if they are the result of low-level contamination, it is also possible that
462 β introgressed into *D. paulistorum* after the two species diverged. We note that β is present

463 in 12 of the 14 sampled lines of *D. paulistorum* and four of its six semispecies. Thus, it is
464 more parsimonious to assume that it was introgressed into *D. paulistorum* once, before
465 semispecies diversification, rather than multiple times throughout evolution. Consequently,
466 β must have been lost and replaced or failed to establish itself in the *D. paulistorum* lines
467 which currently do not carry it i.e., C2 and MS. The introgression of β prior to *D. paulistorum*
468 semispecies divergence is further supported by the similarity in nucleotide composition of
469 the third codon position between the α and β mitotypes. Regardless of whether this
470 convergence is caused by exposure of the β mt-genome to the mutational bias
471 (amelioration) or the selective pressure (codon usage) of *D. paulistorum*, the effect is
472 expected to be stronger when the genomes have been in the same host for a significant time
473 (Marri and Golding, 2008).
474 One question that remains is which forces led to the successful establishment of the
475 β mitotype in *D. paulistorum* and the replacement of its former mitotype. Since *D.*
476 *paulistorum* originally had an α-like mitochondrion and considering that the genomes of
477 α and β are relatively distinct from each other (2.4% divergence), it is likely that the
478 introgression of β would have generated nuclear-mitochondrial incompatibilities (Burton et
479 al., 2013). Such mitonuclear conflicts would in many cases lead to hybrid inviability, lower
480 fitness or selection for the introgressed mitotype to be purged from the population (Burton et
481 al., 2013). However, the β mitotype successfully established itself and spread in *D.*
482 *paulistorum.* This suggests that the β mitotype either provided a strong fitness advantage in
483 the conditions that prevailed at the time of the introgression or that its spread was driven by
484 another factor, such as the endosymbiotic bacterium *Wolbachia* (Dean et al., 2003; Hill,
485 2019). *Wolbachia* strains that show strong reproductive manipulation or that increase host
486 fitness sometimes spread fast through populations and may lead to hitch-hiking of mitotypes
487 associated with infected females (Hurst and Jiggins, 2005; Turelli et al., 1992). In *D.*
488 *paulistorum*, *Wolbachia* has been shown to affect fitness, mate choice and fecundity of some
489 lines (Miller et al., 2010; Schneider et al., 2019). However, it is yet unknown if such effects
490 occur in all semispecies or if they are directly associated with the spread of particular
491 mitotypes in the species.

492 **4.2. Mitochondrial introgressions between semispecies of *D. paulistorum***

493 Apart from the ancestral acquisition of the α and β mitotypes, *D. paulistorum* also shows
494 signs of more recent mitochondrial introgressions across semispecies. These are indicated
495 by incongruences between the mitochondrial and nuclear phylogenies. Given that *D.*
496 *paulistorum* semispecies presently show pre- and post-mating incompatibilities with each
497 other, it is likely that hybridization between them happened in an earlier evolutionary period
498 when such barriers were less developed (Ehrman and Kernaghan, 1972; Ehrman and

499 Powell, 1982). Potentially, sporadic hybridization may still occur in crosses involving the TR
500 semispecies or populations of other semispecies which were shown to be more permissive
501 under lab conditions (Dobzhansky and Pavlovsky, 1967; Ehrman, 1962; Malogolowkin et al.,
502 1964). It is unknown if or how often *D. paulistorum* semispecies hybridize in the wild, but the
503 fact that multiple semispecies can be found in sympatry shows that such events are not
504 frequent enough to blur the distinctions between them (Perez-Salas et al., 1970).
505 We also observe some differences between our results and those of previous studies
506 with regards to which mitotype is associated with certain semispecies, as inferred by an
507 analysis of published phylogenetic trees. While here we see the IN semispecies carrying the
508 β mitotype, it was previously associated with the α mitotype (Gleason et al., 1998) or with
509 both α and β (Robe et al., 2010; Zanini et al., 2018) depending on the mitochondrial marker
510 which was analyzed. Similarly, in this study, the TR semispecies carries the β mitotype, but it
511 was previously associated with the α (Zanini et al., 2018) or with both the α and β mitotypes
512 (Robe et al., 2010) depending on the marker used. Finally, while in our study the AB
513 semispecies carries the α mitotype, it was associated with the β (Gleason et al., 1998; Zanini
514 et al., 2018) or with both the α and β mitotypes (Robe et al., 2010) in previous studies. We
515 note that the IN and TR lines used in the three mentioned studies were collected in the same
516 sampling localities as the ones that we use in the present work, which suggests they may
517 derive from the same lines. The same is true for the AB line used here and in Gleason et al.
518 (1998) and Robe et al. (2010). However, the AB line used in Zanini et al. (2018) was
519 collected in a different location — Santa Catarina, Brazil, while ours is from Mesitas,
520 Colombia. Furthermore, all reference lines that were assayed in the three independent
521 studies were obtained from different laboratories and at different time points. Thus,
522 assuming that semispecies classifications are correct in all studies and that neither
523 contamination nor permutation has occurred in any of the lines, we have two hypotheses to
524 explain the conflicting mitotype associations. One possibility is that the incongruences are
525 due to different populations or individuals from the same semispecies carrying distinct
526 mitotypes i.e., some carry the α and some the β mitotype. Based on our nuclear phylogeny,
527 we do not see evidence of intra-semispecies mitotype variation in our dataset. However, a
528 wider sampling of lines and populations from each semispecies is required to conclude if
529 such variation may occur. Alternatively, the differences between studies may derive from
530 accidental amplification of NUMTs instead of actual mt-genome sequences. Amplification of
531 NUMTs is a known issue when working with mitochondrial data and may misguide
532 phylogenetic and barcoding analyses (Nacer and Raposo do Amaral, 2017; Song et al.,
533 2008). Given this potential pitfall, we advise against using only mitochondrial markers for
534 assigning *D. paulistorum* individuals and lines to semispecies. We also suggest that care

535 should be taken when using solely mitochondrial markers to investigate poorly known
536 lineages in general.

537 5. CONCLUSION

538 In the present study, we use whole genome sequencing combined with phylogenetics and
539 comparative analyses to investigate the mitochondrial evolutionary history of *D. paulistorum*
540 and the willistoni group. We discover that *D. paulistorum* mitochondria are polyphyletic,
541 forming two distinct lineages, and find evidence that neither mitotype is original to the
542 species. The multiple mitochondrial introgression events identified suggest that hybridization
543 between *D. paulistorum* semispecies and across species of the willistoni group have
544 occurred relatively frequently, both recently and in a more distant past. These conclusions
545 are congruent with the recent evolutionary divergence of these lineages as well as their
546 ongoing speciation and underline their value as models in hybridization and speciation
547 studies. Our work also highlights the importance of genomic tools for elucidating past
548 evolutionary and ecological events that contributed to forming the present biodiversity and its
549 genetic variability.

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561 DATA AVAILABILITY

562 Raw sequence data used in this project as well as assembled whole mitochondrial genomes
563 are available in NCBI under the Bioproject PRJNA643793. Accession numbers of individual
564 mitochondrial genomes are listed in **Table S1**.

AUTHOR CONTRIBUTIONS

Guilherme Baião: Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization; Daniela Schneider: Investigation; Wolfgang Miller: Conceptualization, Resources, Funding acquisition, Writing - review & editing, Supervision, Project administration; Lisa Klasson: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

DECLARATION OF COMPETING INTERESTS

The authors declare that they have no competing financial or personal relationships that could have influenced the work reported in this paper.

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