Title:

Persistence and invasiveness of high-level heteroplasmy through biparental transmission of a selfish mitochondrion in *Drosophila*

Running title: Biparental transmission of a selfish mitochondrion in *Drosophila*

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ABSTRACT

Heteroplasmy is the coexistence of more than one type of mitochondria in an organism. Although widespread sequencing has identified several cases of transient or low-level heteroplasmy that primarily occur through mutation or paternal leakage, stable, high-titer heteroplasmy remains rare in animals. In this study we present a unique, stable and high-level heteroplasmy in male and female flies belonging to the neotropical *Drosophila paulistorum* species complex. We show that mitochondria of *D. paulistorum* are polyphyletic and form two clades, α and β, with two subclades each. Mitochondria of the α2 subclade appear functional based on their genomic integrity but are exclusively found in heteroplasmic flies and never in homoplasmy, suggesting that they are a secondary mitotype with distinct functionality from the primary mitochondria. Using qPCR, we show that α2 titer do not respond to energetic demands of the cell and are generally higher in males than females. By crossing hetero- and homoplasmic flies, we find that α2 can be transmitted to their offspring via both parents and that levels are dependent on nuclear background. Following α2 mitotype levels during embryogenesis, we demonstrate that this secondary mitotype replicates rapidly just after fertilization of the egg in a period when primary mitochondria are dormant. This so-called "Replication precox" mitochondrial phenotype likely prevents the α2 mitotype from being outcompeted by the primary mitotype – and thereby secures its persistence and further spread as a selfish mitochondrion, we hereby designate "Spartacus". Finally, we reconstruct the evolutionary history of mitochondria in the willistoni subgroup uncovering signs of multiple mitochondrial losses and introgressions. Our data indicate an α-like mitochondrial ancestor in the willistoni subgroup, with the β mitotype likely acquired via introgression from an unidentified donor. We hypothesize that the selfish characteristics of α2 might have emerged as a response to competition for inheritance with the introgressed β mitotype.

53 **INTRODUCTION**

54 It is generally assumed that individuals of most animal species carry a single, maternally
55 inherited mitochondrial haplotype (a mitotype) [1, 2]. However, recent advances in sequencing
56 technology and genomic analyses show that mitochondrial heteroplasmy, the presence of
57 multiple mitotypes in one individual, is found in a number of vertebrate and invertebrate
58 species [3, 4].

59 Most often, heteroplasmy is the result of either mitochondrial mutations that
60 accumulate over an individual's lifetime, or paternal leakage. In both cases, the additional
61 mitotype is usually transient and persists at low levels within an individual [5]. Most host-
62 mitochondria relationships are ancient, with both parties coevolving over extended periods of
63 time, and disturbances caused by the introduction and persistence of new mitotypes may
64 reduce fitness or cause disease [6, 7]. Stable, long-term heteroplasmy is therefore relatively
65 uncommon and may be evolutionarily disadvantageous (Reviewed in [8]). Accordingly,
66 selection against heteroplasmy has been proposed as an explanation for the evolution of
67 uniparental inheritance in mitochondria [9]. Mechanistically, transmission of male
68 mitochondria in animals is prevented by both pre- and postmating defense and destruction
69 mechanisms of the host ([5, 10-15] and references therein), and if these fail, the rare paternal
70 mitotype is usually outcompeted by maternal mitochondria during early development or within
71 subsequent generations [16].

72 The best-known case of animal heteroplasmy is that of freshwater mussels of the
73 family *Unionidae*, which display double uniparental mitochondrial inheritance (DUI). In these
74 species, two different mitochondrial haplotypes, M and F, are transmitted exclusively by males
75 and females, respectively [17, 18]. Stable heteroplasmy is also found in several isopods in
76 which mitochondria encode different tRNA variants [19-21]. Among insects, prevalence (the
77 frequency of heteroplasmic individuals in a population) at various levels (the proportions of
78 different mitotypes within an individual) has been described in natural populations of the bed
79 bug *Cimex lectularius* L. [22], the neotropical ants of the *Ectatomma ruidum* complex [23], the
80 brown dog tick *Rhipicephalus* spp. [24], and the leaf beetles *Gonioctena intermedia* [25]. For
81 the genetic model system *Drosophila*, natural heteroplasmy has been detected in *D.*
82 *mauritiana* [26], *D. simulans* [27-29], *D. melanogaster* [30], and *D. subobscura* [31, 32].
83 Slightly higher prevalence and levels have been found in interspecies F_1 hybrids of different
84 closely related *Drosophila* species ([33-35] and references therein). In spite of a growing
85 number of reports on heteroplasmy in animals including humans (recently reviewed in [36]),
86 little is known as to how heteroplasmic systems have evolved and what the functional
87 differences between mitotypes could be (recently reviewed in [37]).

88 However, when discussing causes and consequences of mitochondrial heteroplasmy,
89 it might be useful to consider mitotypes as primary or secondary endosymbionts, similarly to

what is done for bacterial endosymbionts of insects where multiple infections and obligate mutualism are common phenomena. Hence, primary bacterial endosymbionts as well as mitochondria are normally maternally inherited, long-term co-evolved with the host and have a well-defined function that make them obligate for the eukaryotic host cell, while secondary bacterial endosymbionts and recently acquired or evolved mitotypes are non-fixed, can have variable, often condition-specific and sometimes harmful effects on their hosts [38-40]. While primary endosymbionts might evolve from secondary endosymbionts as a way for the host to secure higher fitness over long evolutionary time, secondary symbionts can be maintained either because they provide a benefit under specific conditions or because they have the ability to cheat the host and be transmitted selfishly without any positive effect [40]. Similarly, secondary mitochondria might thus be maintained in the host for the same reason and result in heteroplasmy (reviewed in [37]). Additionally, obligate dependencies on multiple endosymbionts evolved in several insect. In such cases, one symbiont has often lost genes so that the genome of the other contain complementary genes involved in a function that was previously performed by only one of them [41, 42]. Hence, similarly, stable heteroplasmy might also be maintained if mitotypes with complementary gene sets are present in the same host.

The Neotropical *Drosophila willistoni* group has been a model for evolutionary studies since the 1950s [43]. Within it, species belonging to the willistoni subgroup have received the most attention, with *Drosophila paulistorum,* in particular, being the focus of many studies on speciation and hybridization [44, 45]. *D. paulistorum* is a complex of six semispecies under incipient speciation, namely Amazonian (AM), Andean Brazilian (AB), Centro-American (CA), Interior (IN), Orinocan (OR) and Transitional (TR) [46], each displaying partially overlapping geographical distribution and varying degrees of pre- and postmating reproductive incompatibilities with the others ([44], and references therein). All semispecies also carry the bacterial endosymbiont *Wolbachia*, which provides mutualistic functions and causes premating isolation between members of different semispecies pairs [47-51]. The whole willistoni subgroup is a recent radiation and phylogenetic incongruences between *D. paulistorum* nuclear and mitochondrial evolutionary histories [52-54] suggest that hybridization and introgression events between semispecies may have occurred in the past [53].

In the present study, we discovered and investigated the unusual biparental inheritance of mitochondria resulting in stable and high-level heteroplasmy in *Drosophila paulistorum*. We assembled the mitochondrial genomes of six species of the willistoni subgroup and three representatives of the closely related saltans species group. We discovered that *D. paulistorum* lines carry mitochondria from two non-sister clades - α and β - and identified several lines exhibiting stable heteroplasmy. We further determined that both mitotypes can be divided into two subclades, called 1 and 2, where the α2 mitotype is so far observed exclusively in heteroplasmic lines. This finding implies that α2 is unable to provide full mitochondrial functionality, and might therefore be regarded as a secondary mitotype, in

128 contrast to the fully functional primary mitotypes (α1, β1 and β2), which can exist in
129 homoplasmy.
130 In addition, the α2 mitotype was found in 41% of the tested *D. paulistorum* lines, where
131 it is stably maintained at variable but significant levels with up to 66% depending on host
132 background. Unexpectedly, this secondary mitotype, unlike the other primary mitochondria we
133 investigated, was not distributed within the fly in a way that correlated with the expected
134 OXPHOS-mediated ATP demands of different tissues. Yet, the α2 genome showed no signs
135 of deterioration, suggesting it may perform some unknown function for the host. Additionally,
136 we demonstrated that α2 mitochondria are biparentally transmitted and start replicating rapidly
137 after egg fertilization, while the primary mitochondria are still quiescent. This replication
138 advantage, which we name the "Replication precox" phenotype, allows the α2 to reach high
139 levels during early embryogenesis and thus to avoid being outcompeted by the much larger
140 number of maternally inherited primary mitochondria. Hence, we propose that α2 is a newly
141 discovered selfish mitotype that can persist and spread in and between populations, and
142 possibly even across species by introgression, via the combination of biparental transmission
143 and the Replication precox phenotype.
144 Using phylogenetic reconstruction, we observed a complex evolutionary history of
145 mitochondria in *D. paulistorum*, with both ancient and recent introgressions. We suggest that
146 an α-like mitochondrion was the ancestral mitotype of the species and that heteroplasmy might
147 have originated through introgression of the β mitotype from an unsampled or extinct donor,
148 a concept known as 'ghost' introgression [55-57]. We speculate that the unusual biparental
149 transmission mode of α2 might have developed through competition with the β mitochondria
150 for maternal transmission.
151

152 # RESULTS

153 **High level heteroplasmy is common in the *D. paulistorum* species complex**

154 In the course of our phylogenetic survey we sequenced PCR products of the mitochondrial
155 *Cytochrome Oxidase 1* (*CO1*) gene from several *D. paulistorum* lines for typing and found
156 multiple robust double peaks in the resulting chromatograms derived from single flies of the
157 Orinocan O11 line. The double peaks indicated the presence of two bases within a diagnostic
158 *Hin*dIII restriction site (**Fig 1A**). Thus, to independently confirm heteroplasmy, we performed
159 diagnostic RFLP/*Hin*dIII Southern blots on single fly DNA probed with *CO1*. We found that the
160 O11 line is truly heteroplasmic, with individual flies carrying two mitotypes, hereafter called α
161 and β, at approximately similar levels (**Fig 1B**). In contrast, single flies of the Centro-American
162 C2 and Amazonian A28 lines were homoplasmic and carried either the α or β mitotype,
163 respectively (**Fig 1B)**. By using *CO1*-specific RFLP/*Hin*dIII-PCR we further showed that all

tested O11 flies (20 females, 20 males) exhibited both the α and β specific bands (**Fig 1C**), which suggested fixation of the high-level heteroplasmic state in this *D. paulistorum* long-term lab line.

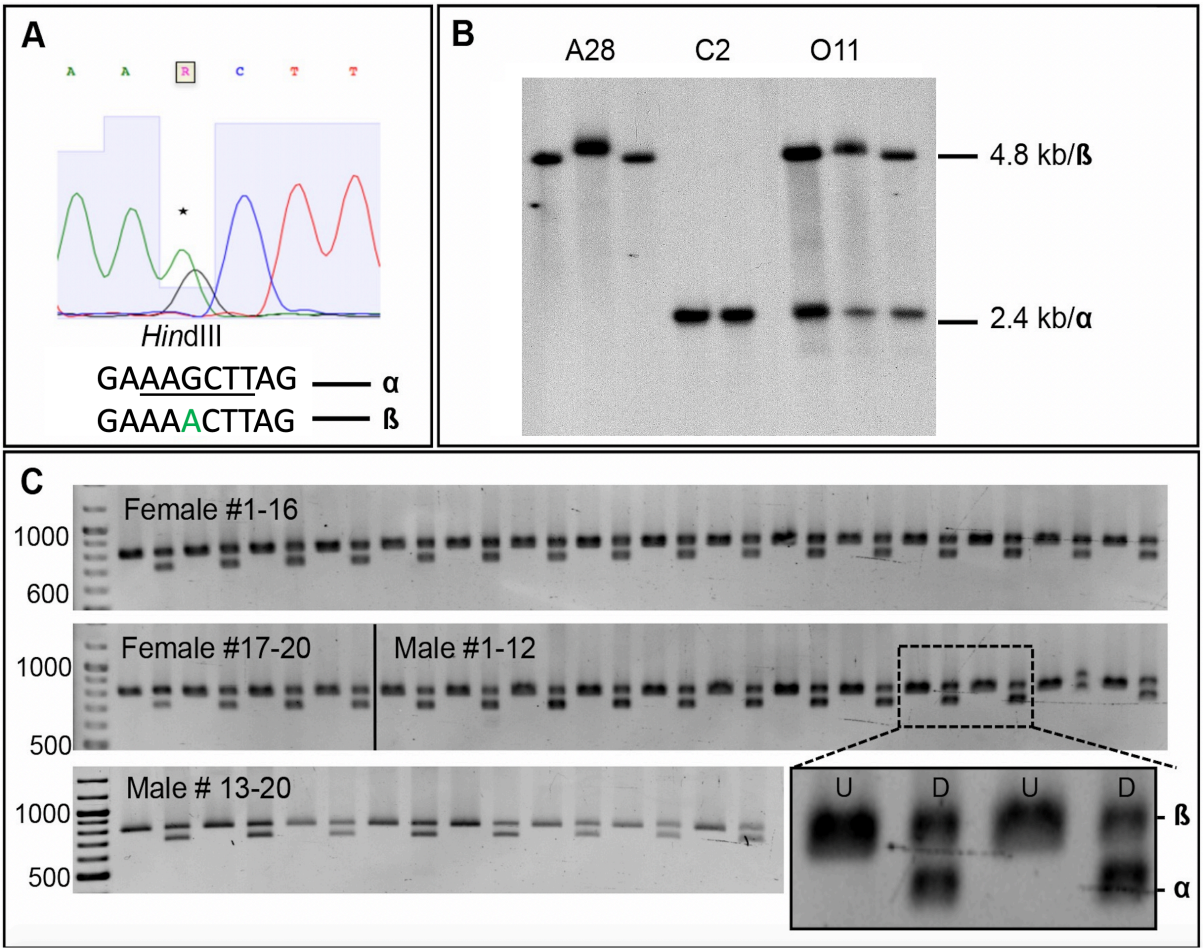

Fig 1. High-level heteroplasmy in the *D. paulistorum* Orinocan line O11. **(A)** Chromatogram derived from *CO1* direct Sanger sequencing of one O11 female covering the α-diagnostic *Hin*dIII restriction site (underlined). The asterisk highlights the double peak with Cytosine (C) for α and Thymine (T) for β-mitotype. **(B)** Single-fly *CO1* Southern blot digested with *Hin*dIII. While the Amazonian females of the A28 line (n=3) are homoplasmic for the β-mitotype (4.8kb) and the ones of the Centro-American line C2 (n=2) are homoplasmic for α (2.4kb), all randomly picked Orinocan O11 females (n=3) show high-level heteroplasmy of the α- and β-mitotypes (4.8 and 2.4kb). **(C)** α-diagnostic *CO1* RFLP/*Hin*dIII-PCR screen on O11 single flies (n=20 for each sex). Undigested (U) and digested (D) *CO1*-PCR fragments per fly are shown (bottom right in **C**). As all tested single flies (40/40) exhibited clear double bands after *Hin*dIII digestion, hence fixation of the α/β heteroplasmy in the O11 line was assumed.

Whole-genome sequencing shows widespread heteroplasmy forming two distinct mitochondrial clades and four subclades in D. paulistorum

Next, we used Illumina sequencing to screen for heteroplasmy on a genome-wide level in *D. paulistorum* and several related species. We sequenced a total of 23 *Drosophila* lines representing six species of the willistoni group and three species of the closely related saltans group (**Fig 2A, Table S1**). Thirteen *D. paulistorum* lines were sequenced, including representatives of the six classical semispecies and seven recently collected isofemale lines

from Brazil and French Guiana (**Table S1**). Five of the sequenced *D. paulistorum* lines (38.5%) were found to be heteroplasmic, i.e., the Orinocan FG103, FG111 and O11 lines, the Amazonian FG572 line and the Andean-Brazilian MS line (**Fig 2B**). Importantly, heteroplasmy was not detected in any other tested neotropical species apart from *D. paulistorum*.

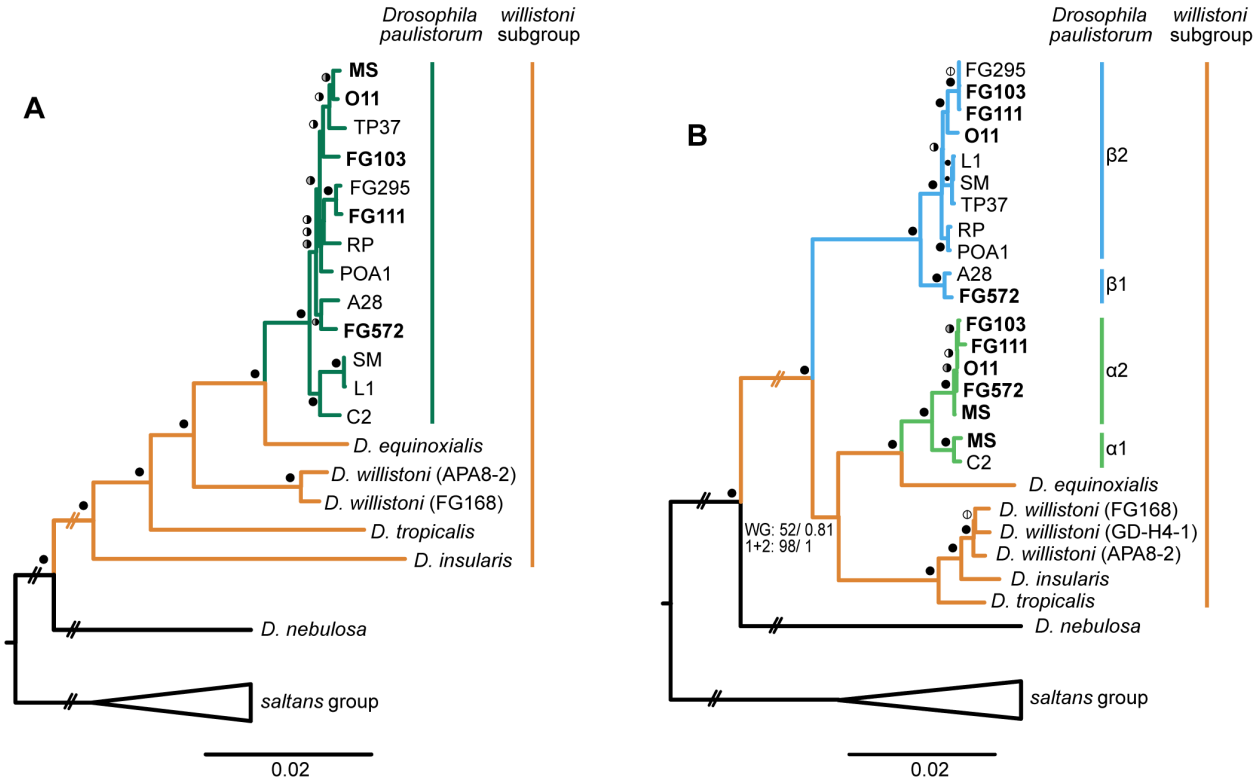

Fig 2. Phylogenies of the sequenced *Drosophila* lines. Trees based on (**A**) the nuclear genes *28S*, *Adh*, *CAD/r*, *Ddc*, *Hb* and *Per*; and (**B**) whole mitochondrial genomes. Fly lines in bold are heteroplasmic. *D. paulistorum* mitochondria split into two distinct non-sister clades: α (green) and β (blue). Support for the branch defining α as sister clade to other species of the willistoni subgroup is low for whole genome (WG) phylogeny, but high when only first and second codon positions of protein coding genes are analyzed (1+2). The same topologies are obtained by maximum likelihood (GTRGAMMA model) and Bayesian analyses. Colored circles next to the nodes indicate ML bootstrap support (left half) and Bayesian posterior probability (right half). Black corresponds to 99-100% bootstrap support or 0.99-1.0 posterior probability, grey represents 70-98% bootstrap support or 0.7-0.98 posterior probability, white indicates support below 70%. Branches leading to the outgroups and marked by two transverse lines were shortened and don't follow the presented scale.

Among all sequenced lines of willistoni and saltans group species, a total of 28 mitochondrial genomes were assembled, one for each homoplasmic *Drosophila* line and two for each of the heteroplasmic *D. paulistorum* lines (see Methods). The genomes are approximately 16.000 bp long, have a GC content around 21% (**Table S2**), and encode the same 13 proteins, 22 tRNAs and two rRNAs as the mitochondria of *D. melanogaster* and most other animals. Importantly, no loss-of-function mutations were found, indicating that all genomes are likely to encode fully functional mitochondria. The assembled mitochondrial genomes also show considerably higher read coverage than nuclear contigs from the same lines, which

214 distinguishes them from potential nuclear mitochondrial DNA sequences (NUMTs) (**Table S3**).
215 All of the assembled mitochondrial genomes have short control regions (CR), between 1.1
216 kbp in the willistoni group and 1.3 kbp in the saltans group, compared to the 4.6 kbp CR in *D.*
217 *melanogaster*. A few substitutions occur between *D. paulistorum* lines in or near the putative
218 origins of replication, but no major differences in length are observed between CRs (**Table S2**,
219 **Fig S1**).
220 Phylogenetic analysis of the assembled genomes revealed that the mitochondria of *D.*
221 *paulistorum* are polyphyletic and split into two major clades (**Fig 2B**), the α and β mitotypes
222 that were previously detected in single flies of O11 (**Fig 1**). The mitochondrial genomes of the
223 α and β clades are relatively distantly related to each other with an average nucleotide
224 divergence of 2.94%. A majority of the substitutions that occur between them in protein coding
225 genes are synonymous (**Tables S4, S5**), indicating that purifying selection is acting on the
226 genomes of both clades. Based on the phylogeny, the α and β clades can be further divided
227 into two subclades, named 1 and 2 **(Fig 2B**). Interestingly, while the α1, β1 and β2 mitotypes
228 occur both in homoplasmic and heteroplasmic lines, the α2 mitotype is never found in
229 homoplasmy and is present in all heteroplasmic lines, where it is partnered with a mitotype
230 from another subclade.
231
232 *Level and prevalence of heteroplasmy in D. paulistorum lines*
233 To further investigate the heteroplasmy of *D. paulistorum*, we assessed its prevalence (the
234 percentage of heteroplasmic flies within a line) and levels (percentage of each mitotype within
235 a sample) using mitotype-specific RFLP-PCR. Seventeen lines belonging to the six *D.*
236 *paulistorum* semispecies were tested and seven (41%) displayed heteroplasmy (**Table 1**).
237 Apart from the lines already identified as heteroplasmic through genome sequencing (**Fig 2B)**,
238 we further identified heteroplasmy in the two Amazonian lines FG16 and FG572 as well as in
239 the Andean-Brazilian Yellow lines. Interestingly, the Orinocan FG103 line was clearly
240 heteroplasmic at the time of sequencing, shortly after its field collection and the establishment
241 of the isofemale line in 2014/2015 (**Table 1**), but thereafter lost the α2 mitotype in consecutive
242 generations. Heteroplasmic lines were thus observed in the Amazonian, Andean-Brazilian and
243 Orinocan semispecies and originate from both long-term lab lines and recently collected field
244 samples (**Table S1**).
245
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Table 1. Levels of α2 and prevalence of heteroplasmy in *D. paulistorum* lines based on Illumina sequencing of ovary samples (α2 level Illumina) and RFLP-PCR from adult flies (α2 level RFLP**)**. A dash (-) in the α2 level from Illumina sequencing means that we are unable to determine that the α2 mitotype existed. Levels of α2 obtained through RFLP-PCR are presented separately for females (left) and males (right). Prevalence of heteroplasmy among tested individuals (Het. prevalence) is presented both as a percentage and in absolute numbers (in parenthesis). Abbreviations: AB: Andean-Brazilian, AM: Amazonian, CA: Centro-American, Coll: Collection year, Het: heteroplasmy, IN: Interior, ND: Not determined, OR: Orinocan, Spp: Semispecies, TR: Transitional. Further details about the fly lines are provided in **Table S1**.

Line	Coll.	Spp.	Mitotype	α2 level Illumina	α2 level RFLP	Het. prevalence
O11	1957	OR	α2/ß2	19.4%	30.7±3.7/36.8±3.6	100% (39/39)
FG103	2014	OR	α2/ß2	6.5%	ND§	0% (0/40)§
FG111	2014	OR	α2/ß2	2.3%	13.4±1.1/18.3±2.9	25% (10/40)
FG295	2014	OR	ß2	-	0	0% (0/40)
TP37	2009	OR	ß2	-	0	0% (0/20)
POA1	2003	OR	ß2	-	0	0% (0/20)
RP	1995	OR	ß2	-	0	0% (0/20)
A28	1952	AM	ß1	-	0	0% (0/40)
FG16	2018	AM	α2/ß1	ND	57.2±8.6/66.5±7.2	55% (22/40)
FG18	2018	AM	ß1	ND	0	0% (0/40)
FG572	2015	AM	α2/ß1	49.5%	45.3±8.1/63.7±7.0	90% (36/40)
MS	1962	AB	α1/α2	19.1%	21.8±3.1/36.0±4.1	100% (40/40)
Yellow	1960	AB	α1/α2	ND	20.4±4.7/27.4±4.3	100% (40/40)
C2	1954	CA	α1	-	0	0% (0/20)
White	1960	CA	α1	ND	0	0% (0/20)
L1	1958	IN	ß2	-	0	0% (0/20)
SM	1956	TR	ß2	-	0	0% (0/20)

§The line FG103 was heteroplasmic in 2014/15 (determined by Sanger and Illumina sequencing), but has lost the α2 mitotype afterwards (verified by RFLP-PCR and Sanger sequencing in 2018/19).

Whereas heteroplasmy is fixed in the three independent long-term lab lines O11, MS and Yellow, α2 prevalence ranges between 25% and 90% in the recently collected isofemale lines FG111, FG16 and FG572 (**Table 1**). Levels of α2 were high within individual flies of most lines (**Table 1, Fig S2)** and appear to be associated with the nuclear background of the host. The three heteroplasmic lines of the Orinocan semispecies, FG103, FG111 and O11 displayed different α2 levels (**Table 1**). However, the two lab lines of the Andean-Brazilian semispecies (MS, Yellow) showed levels of α2 that were similar to each other at 20%. Finally, the highest α2 levels (45-60%) were detected in the heteroplasmic lines of the Amazonian semispecies, FG16 and FG572 (**Table 1, Fig S2**). In addition, we found that levels of α2 were significantly higher in male flies compared to females in all tested lines except for FG111 ($p<0.001$ for FG572, MS, $p<0.01$ for Yellow and FG16, $p<0.05$ for O11, Tukey HSD test, **Fig S2; Supplemental data file**). However, since α2 levels were measured as a ratio between mitotypes, it is not clear if the higher level in males results from fluctuations in copy numbers of α2, of the primary mitotype (α1, β1 or β2), or of both.

280

281 **Titers of α2 and β2 mitotypes in *D. paulistorum* tissues**

282 To explore the dynamics and potential function of α2 and β2 mitochondria, we measured the
283 genome copy numbers of each mitotype relative to nuclear genome copies (from here on
284 referred to as titer) in the heteroplasmic O11 line of the Orinocan semispecies. Since
285 mitochondrial copy numbers are variable in different tissues and under different conditions,
286 their location and titer can provide clues as to their function. Hence, besides whole flies we
287 also investigated α2 and β2 titers in the head, thorax, abdominal carcasses without gonads
288 and gonads of male and female O11 flies using discriminant qPCR. We also explored the
289 potential influence of sex, age, and infection with the endosymbiont *Wolbachia* on titers of
290 both mitotypes. We found that titers of the primary β2 mitotype (**Fig 3A**) were significantly
291 influenced by tissue ($F_{4,80}$=25.44, p<0.001). As expected, β2 is found in higher titers in energy-
292 demanding tissues where ATP is provided by oxidative phosphorylation (OXPHOS) such as
293 in the flight muscle of the thorax ([5], and references therein). Further, we found a weak
294 significant interaction between age and sex ($F_{1,80}$=6.12, p<0.05) potentially driven by younger
295 male flies having slightly more β2 mitochondria than older males, in combination with different
296 tissue location patterns between the sexes. Females had overall lower numbers of the primary
297 β2 mitotype per nuclear genome copy than males ($F_{1,80}$=28.68, p<0.001).

298 In contrast to β2, we found no significant effect of fly tissue ($F_{4,80}$=1.426, p=0.23) or
299 age ($F_{1,80}$=0, p=0.99) on the titer of α2. Additionally, there was no significant effect of
300 *Wolbachia* infection on the titer of either the β2 ($F_{1,80}$=1.41, p=0.24) or the α2 ($F_{1,80}$=0, p=0.97)
301 mitotypes. The copy number of α2 is, however, significantly affected by fly sex (**Fig 3B**,
302 $F_{1,80}$=51.34, p<0.001), with males carrying approximately twice as many α2 copies as females
303 per nuclear genome. Significant higher titers of α2 in O11 males corroborate our finding of
304 generally higher α2 levels in heteroplasmic males compared to heteroplasmic female flies
305 (**Table 1; Fig S2** and **Supplemental Data file**), confirming that the significant differences
306 between the sexes in the percentage data are due to more copies of α2, and not for example,
307 lower β2 numbers.

308 The fact that only the titers of the β2 mitotype varied depending on fly tissue, but not
309 the ones of α2, supports our conclusion that β2 is the functional and hence the primary
310 mitochondrion and that α2 might be dysfunctional and/or not able to generate sufficient energy
311 for its host.

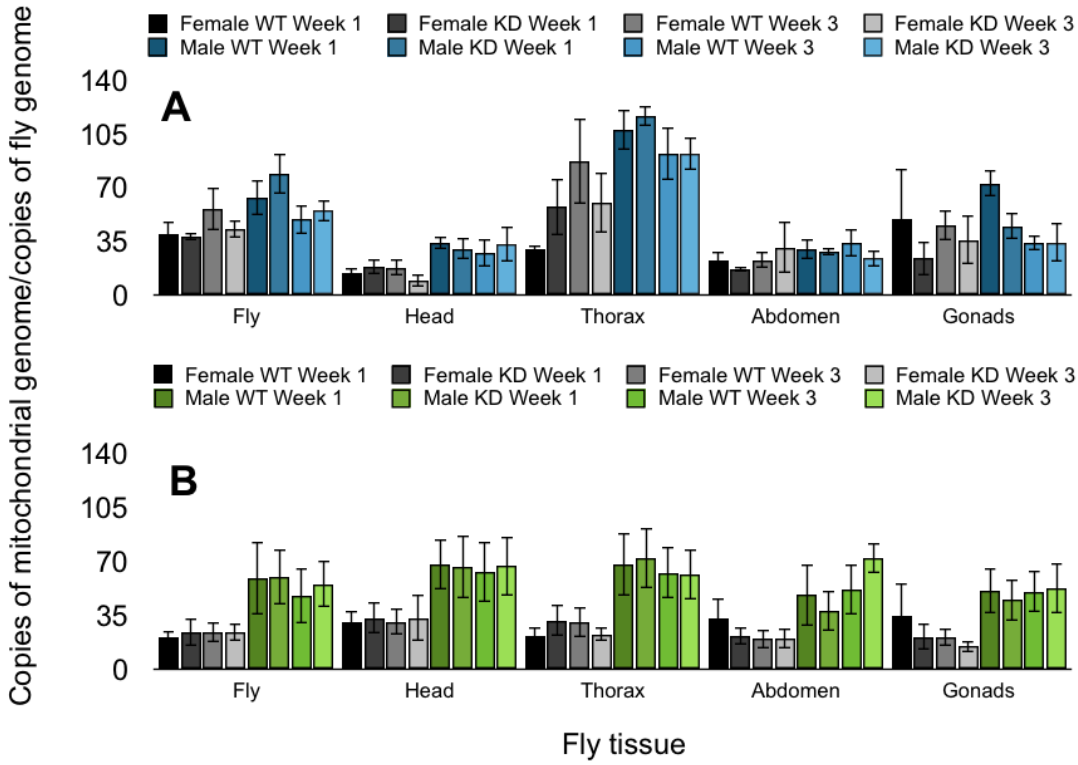


Fig 3. Absolute copies of β2 and α2 mitochondria in O11 *D. paulistorum* flies, relative to fly nuclear genome copy. Bar charts present **(A)** β2 and **(B)** α2 mitochondria in male and female flies, dissected tissues and across two time points (1 and 3 weeks) as well as whether the flies had been treated with antibiotics leading to a knockdown of *Wolbachia*. Abbreviations: WT: wild-type flies. KD: *Wolbachia* knock-down flies. Three biological repeats are plotted with error bars of 1 standard error.

Biparental transmission and levels of the α2 mitotype in intra- and inter-semispecies hybrids of *D. paulistorum*

We used *CO1* RFLP-PCR to investigate if paternal transmission plays a role in the maintenance of high level heteroplasmy in *D. paulistorum*. Relative levels of α2 in F_1 offspring were measured in various crosses involving homoplasmic females and heteroplasmic males in intra- and intersemispecies combinations. Semispecies designation of the tester lines was determined by compatibility crosses (**Table S6**). By this, we uncovered unusual high levels of paternal transmission of α2. In intra-semispecies crosses, all tested F_1 flies in the FG295 (β2) x O11 (β2 and α2) cross (40/40, **Fig 4A**) and 57.5% flies in the A28 x FG572 cross (23/40, **Fig 4B**) were heteroplasmic (Supplemental data for Fig 4). F_1 female hybrids from FG295 x O11 cross had significantly lower α2 levels than O11 females (parental line) ($p<0.001$, Tukey HSD test), whereas the F_1 male hybrids from the same cross as well as both F_1 sexes from the A28 x FG572 cross did not significantly differ in levels from their parental ones of the same sex ($p=0.99$ for former and $p=0.06$ and $p=0.46$ for the latter two, respectively). As

333 heteroplasmy is not fixed in the FG572 line (**Table 1**) paternal introgression in A28 was also
334 incomplete (**Fig 4B).**
335 In inter-semispecies crosses between Amazonian and Orinocan flies all tested F_1 flies
336 of A28 (β1) x O11 (40/40, **Fig 4C**) and 45% flies of FG18 (β1) x O11 (18/40, **Fig 4D**) were
337 heteroplasmic. In both combinations, the α2 mitotype levels of F_1 flies of both sexes were
338 significantly lower than in the paternal O11 line (p<0.001 for both crosses, Tukey HSD test).
339 Combining data from all four crosses (**Fig 4 A-D**), paternal transmission of α2 was found in
340 76% of F_1 hybrids (121/160). This finding clearly demonstrates that the α2 mitochondria of *D.*
341 *paulistorum* are not only sporadically leaking but are highly transmissible via males. To
342 independently confirm these results, we tested a subset of the heteroplasmic F_1 hybrids for
343 their α2 levels using a second mitochondrial locus (*CO2*) and observed patterns similar to
344 *CO1* (**Fig S3**).
345 Additionally, we investigated whether inheritance of paternal mitochondria is α2-
346 specific. For this, we used mitotype-specific RFLP-PCR or Sanger sequencing on F_1 hybrid
347 flies generated by eight different cross combinations aimed at tracing paternal transmission of
348 either α1 (number of F_1 flies assayed = 59), β1 (31), or the β2 (76) mitochondria. Paternal
349 transmission was not detected in any of the 166 tested F_1 flies (**Table S7**). Hence, a high level
350 of paternal transmission is unique for α2 mitochondria.
351 We verified our findings using highly sensitive discriminant qPCR on A28 x O11
352 interspecies crosses. We assessed quantities of the β1, β2 and α2 mitotypes in O11 males
353 (α2 and β2), A28 females (β1) and F_1 hybrid offspring. As expected, the pure parental lines
354 possessed only the expected mitotypes (**Fig 4E, F**). Despite robust paternal inheritance of α2
355 in all tested F_1 hybrids (16/16), the hybrid offspring do not show any significant differences in
356 titers for any mitotype when compared to their homoplasmic mothers, which is likely due to
357 low α2 titers in the hybrids and relatively few biological replicates. However, significantly
358 different titers for all mitotypes were seen when F_1 hybrids are compared to their heteroplasmic
359 fathers **(Fig 4E, F, G**), i.e. higher β1, lower β2 and lower α2 titers are seen in F_1 hybrids
360 compared to the paternal line. In contrast to the less sensitive RFLP-PCR method, our
361 discriminant qPCR method allowed us to detect very low levels (~0.1-0.3%) of paternal β2
362 leakage in 11 out of 16 hybrid offspring (Supplementary data file).
363 As expected for an inter-semispecies cross [44, 45], all F_1 hybrids were sterile and did
364 not produce any live larvae when crossed either to siblings or backcrossed to parental lines
365 (**Table S8**). This prevented exploration of the mitotype dynamics in further generations from
366 this combination of parental lines. We were also unable to explore the dynamics of the reverse
367 cross (female O11 with male A28), and thereby test if the β1 mitotype could be paternally
368 transmitted, as none of the crosses we set up (0/200 attempts) produced larvae.

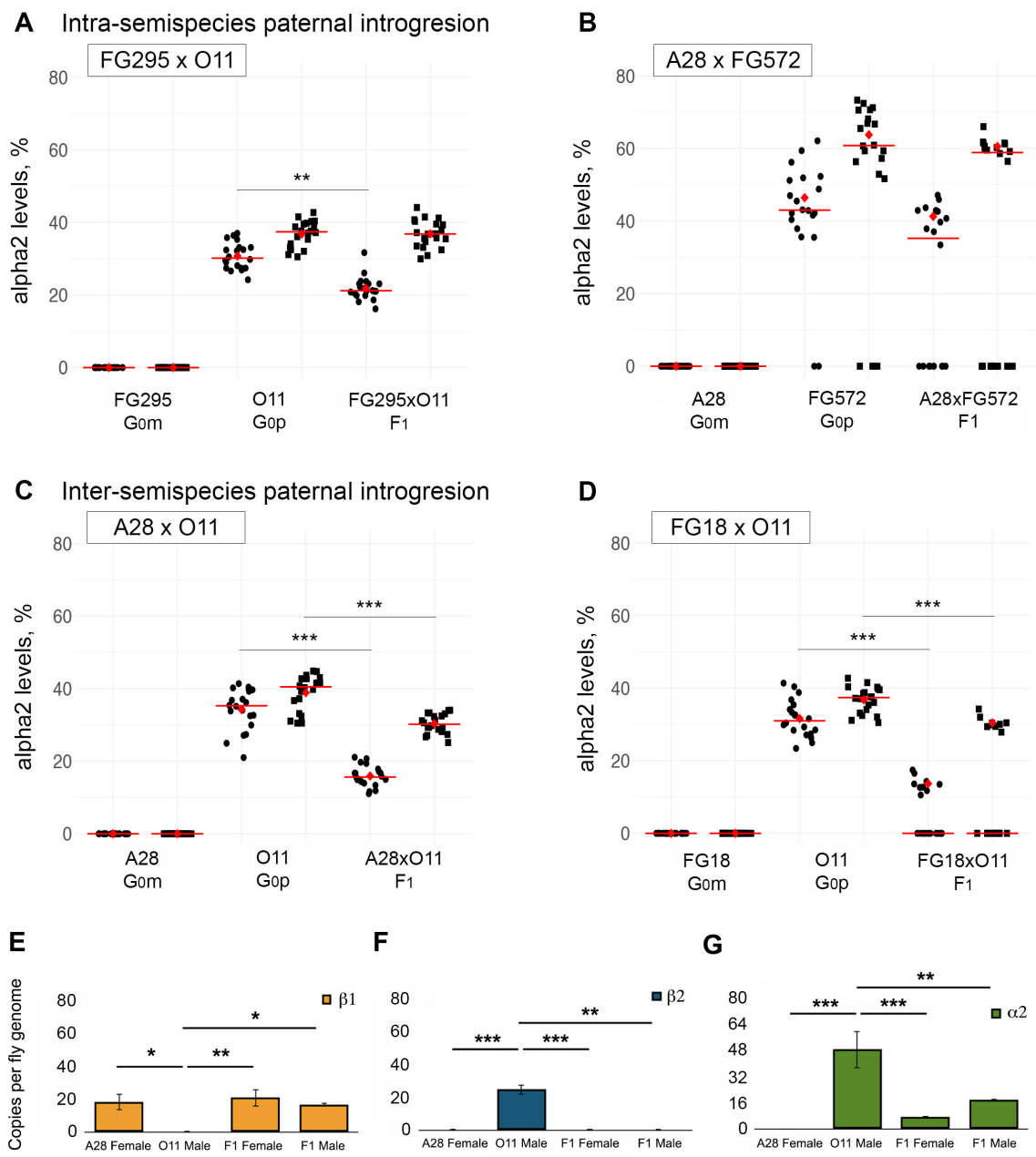


Fig. 4. Levels of the paternal α2 mitotype in intra- and inter-semispecies hybrids of *D. paulistorum*. Paternal inheritance of α2 mitochondria in different *D. paulistorum* hybrids determined by RFLP-PCR **(A-D)**. Scatter plots present relative α2 levels in single fly F_1 hybrids derived from homoplasmic females (circle) and heteroplasmic males (square) of **(A, B)** intra-semispecies (FG295 x O11; A28 x FG572) and **(C, D)** inter-semispecies crosses (A28 x O11; FG18 x O11). Relative levels in F_1 hybrids (F_1 circle, F_1 square) were calculated based on *Hin*dIII-RFLP using *CO1* locus, and compared to the maternal (G_0m fem, G_0m mal) and paternal (G_0p fem, G_0p mal) lines. Single values are plotted including median (red crossbar) of all individuals and mean (red rhombus) of heteroplasmic flies (n=20 per sex, flies were 7-10 days old). Significance of difference between α2 levels was estimated by ANOVA (p<0.05*, p<0.001***). Paternal α2-inheritance determined by discriminant qPCR (**E-G**). Number of copies of β1 (**E**), β2 (**F**) and α2 (**G**) mitochondrial genomes in 3-week old O11, A28 parents and F_1 hybrids relative to fly nuclear genome copy. Data was generated from crosses from single virgin males and single virgin female flies. N=8 in all cases and error bars are 1 standard error. Post-hoc significances are shown as *** = p<0.001, ** = p<0.01, * = p<0.05.

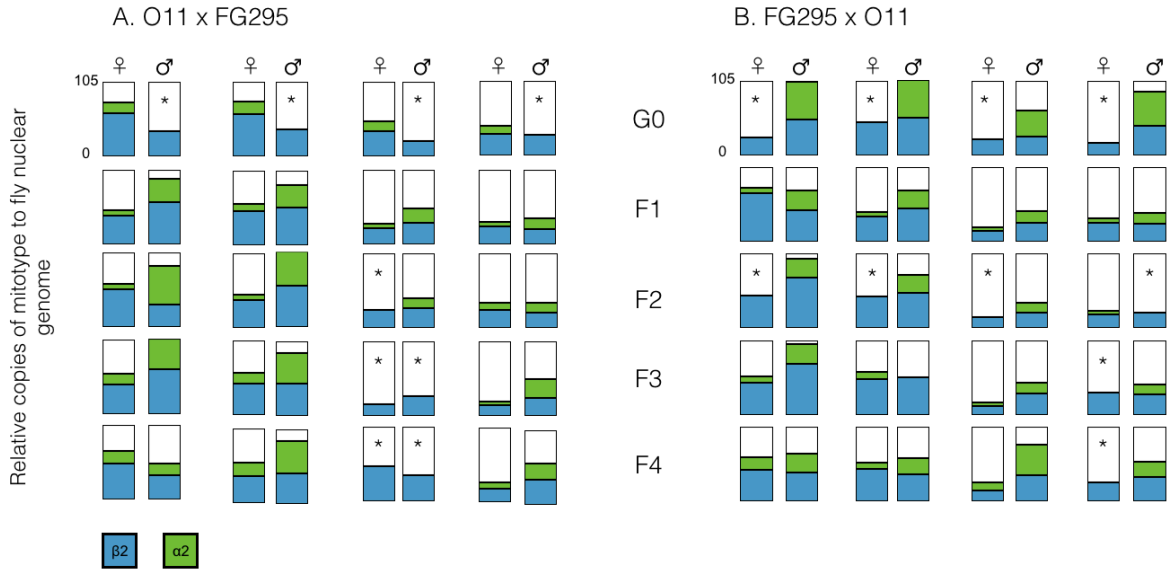
385 **High invasiveness and stable persistence of the α2 mitochondria over multiple**
386 **generations**

387 After identifying that α2 was the only mitotype paternally transmitted in high frequency to F_1
388 flies, we investigated persistence of heteroplasmy over further generations. We used
389 discriminant qPCR to follow the inheritance patterns of the α2 mitotype during four generations
390 of fertile intra-semispecies FG295 (homoplasmic β2) and O11 (heteroplasmic α2 and β2)
391 hybrid crosses. We followed four single-fly G_0 crosses in each direction (FG295 x O11 and
392 O11 x FG295), and mated a randomly picked pair of virgin siblings to each other in each
393 consecutive generation (F_1-F_4) to explore single-fly inheritance patterns of the α2 mitotype
394 (**Fig 5**). The results showed both maternal (**Fig 5A**) and paternal (**Fig 5B**) transmission of the
395 α2 mitotype, as well as high α2 invasiveness (ability to invade a homoplasmic line) and
396 persistence (ability to be maintained over generations) in F_4 flies (13/16).

397 In both crossing directions, all F_1 hybrids (16/16) were heteroplasmic, hence
398 demonstrating not only paternal but biparental transmission of α2. However, in the following
399 generations (F_2-F_4), transmission success was more varied. All tested offspring from three of
400 the four crosses starting with a heteroplasmic mother carried both α2 and β2, and one cross
401 lost the heteroplasmy at F_3 (**Fig 5A**), whereas the crosses starting with a heteroplasmic father
402 showed more stochasticity in transmission, with the α2 mitotype not necessarily transferred to
403 both tested offspring in these later generations (**Fig 5B**). However, more individuals need to
404 be tested in order to confirm this pattern.

405 In agreement with earlier results, we found that there were differences in mitochondrial
406 titers between heteroplasmic and homoplasmic flies, which are dependent on sex. While titers
407 of the β2 mitotype were not significantly different between hybrid males and females across
408 the experiment (Wilcoxon rank sum test W=866, p=0.29), male hybrids had higher titers of the
409 α2 mitotype compared to hybrid females (Wilcoxon rank sum test W=1208, p<0.001). Overall,
410 heteroplasmic flies also carried a higher total mitochondrial load than homoplasmic flies
411 (Wilcoxon rank sum test W=183, p<0.001). After separating the data by the direction of the
412 cross and the sex of the fly, we found that heteroplasmic sons had significantly higher total
413 mitochondrial titers than their homoplasmic parents (Wilcoxon rank sum test W=5, p<0.05 for
414 female parents; W=2, p<0.01 for male parents), while heteroplasmic daughters do not
415 (Wilcoxon rank sum test W=10, p=0.138; W=12, p=0.13 for male parents), demonstrating the
416 noticeably higher titers in male heteroplasmic flies specifically. In contrast, heteroplasmic
417 fathers have significantly higher total mitochondrial titers than their heteroplasmic sons and
418 daughters (sons W=47, p<0.05; daughters W=43, p<0.05), whereas the difference in titer
419 between heteroplasmic mothers and their heteroplasmic offspring is non-significant for either
420 sex (sons W=19, p=0.382; daughters W= 38, p=0.202).

421 Given that we only observe significant variability in the α2 titer between sexes as well
422 as between parents and their offspring (no significant difference was observed for any test on
423 β2), the higher titer of mitochondria in heteroplasmic flies overall suggest that the presence of
424 α2 increases the number of total mitochondria per cell and is added on top of the primary
425 mitochondria, i.e., β2. The reduction in total mitochondrial titer between heteroplasmic fathers
426 of the O11 line and their heteroplasmic offspring of both sexes cannot be explained by the
427 significantly different α2 titers observed between male and female flies. Instead, this result
428 indicates an interaction between mitotype titer and host nuclear background, which is more
429 pronounced in males.



430

431 **Fig 5. Presence and titer of α2 and β2 in (A) O11 x FG295 and (B) FG295 x O11 hybrids.**
432 Relative copies of α2 to number of fly nuclear copies is shown in green. Relative copies of β2
433 to number of fly nuclear copies is shown in blue. Values were rounded up if the decimal was
434 over 0.5 and rounded down if under 0.5. The figure depicts four biological repeats of each
435 direction of the hybrid cross, each representing a single-pair mating of a male and female fly
436 for each line. A mated pair derived from randomly picked virgin flies of each generation was
437 tested for four generations after the initial mating and copy numbers of α2 and β2 mitochondria
438 were quantified through discriminant qPCR. ∗ means that α2 was below the cutoff threshold
439 on qPCR and the fly is considered homoplasmic.
440

441 To further investigate whether fly nuclear background affects the persistence of the α2
442 mitotype we backcrossed virgin F_1 hybrids from O11 x FG295 to the FG295 line (**Fig 6A**),
443 thereby increasing the nuclear background that coevolved with the homoplasmic β2 mitotype
444 in each generation. As before, all F_1 flies acquired the α2 mitotype when transmitted
445 maternally (**Fig 6A**, left column), but their respective levels were lower than in the G_0 parent
446 flies (p<0.001, Tukey HSD test). Backcrossing these F_1 flies to the homoplasmic FG295 line
447 (BC1, maternal introgression) showed a decrease in α2 prevalence in the offspring (**Table S9**).
448 As in F_1 flies, the levels of α2 in offspring from the hybrid parents were significantly lower than

449 in G_0 flies (p<0.001, Tukey HSD test). A second backcross of the BC1 offspring to the
450 homoplasmic FG295 line revealed that α2 prevalence decreased in the BC2 offspring from
451 the BC1 hybrid mothers, whereas their paternal counterparts kept a similar prevalence as that
452 observed in BC1. Overall α2 levels in BC2 flies were significantly lower in both sexes in all
453 backcrossing directions (p<0.001, Tukey HSD test) compared to their parental levels (G_0
454 females - Mean=44.5, SD=4.5; G_0 males - Mean=52.4, SD=4.8).

455 Again, all F_1 flies also inherited the α2 mitotype when initially transmitted paternally
456 (**Fig 6A**, right column). Compared to G_0 flies of the same sex, the levels of α2 were significantly
457 lower in F_1 females but not significantly different in F_1 males (p<0.01 and p=0.56, respectively,
458 Tukey HSD test). Backcrossing of these F_1 flies to the FG295 line (BC1, paternal introgression)
459 showed a decrease in α2 prevalence in the offspring when transmitted both maternally and
460 paternally (**Table S9**). Compared to the F_1 flies of the same sex, the levels of α2 in all BC
461 offspring from both hybrid parents decreased to approximately 20% in females and 30% in
462 males (p<0.001, Tukey HSD test).

463 A second backcross of the BC1 offspring to the FG295 line revealed a pattern similar
464 to the one of the reciprocal O11 x FG295 cross. The prevalence of α2 was low in the
465 backcrossed offspring derived from hybrid mothers whereas the flies from hybrid fathers had
466 a prevalence similar to that observed in BC1. The only exception was the offspring obtained
467 from mothers derived from the F_1 hybrid fathers (40% for females and 40% for males). By
468 increasing the FG295 nuclear background, all BC2 offspring showed significantly lower α2
469 levels (p<0.001, Tukey HSD test; see **Table S9**) compared to the levels in their heteroplasmic
470 O11 parents of the same sex. In summary, we observed that backcrossing heteroplasmic F_1
471 hybrid flies from both crossing directions to a homoplasmic β2 line not only lowers relative α2
472 levels but also results in a decrease in heteroplasmy prevalence in further generations. These
473 data indicate that the α2 mitotype can successfully invade previously homoplasmic lines,
474 although its transmission success is affected by the host nuclear background.

475 Additionally, we investigated the capacity of α2 to invade other species by performing
476 crosses between females of *D. equinoxialis* and *D. willistoni* and males of *D. paulistorum* O11
477 line (**Fig 6B, 6C**). Since hybrid flies from these interspecies crosses are fully sterile, we could
478 not assess α2 prevalence, persistence and levels beyond F_1 flies. Both cross combinations
479 showed 100% prevalence of the α2 mitotype in F_1 offspring, although due to the high
480 embryonic lethality of hybrids we were only able to analyze eight F_1 flies in the *D. equinoxialis*
481 x O11 cross (**Fig 6B**) and two F_1 flies in the *D. willistoni* x O11 cross (**Fig 6C**). In agreement
482 with earlier experiments [58], no F_1 males were obtained in either interspecies crosses, thus,
483 only females were analyzed for mitochondria levels. Similar to results derived from *D.*
484 *paulistorum* inter-semispecies crosses (**Fig 4**) F_1 females of *D. equinoxialis* x O11 cross (**Fig**
485 **6B**) had significantly lower α2 levels in comparison to G_0 flies (11%, p<0.001, Tukey HSD

test). α2 levels in the two F_1 females of *D. willistoni* x O11 cross were also decreased, but due to the extremely low number of viable F_1 hybrid flies (**Fig 6C**) we were not able to analyze significance of this result.

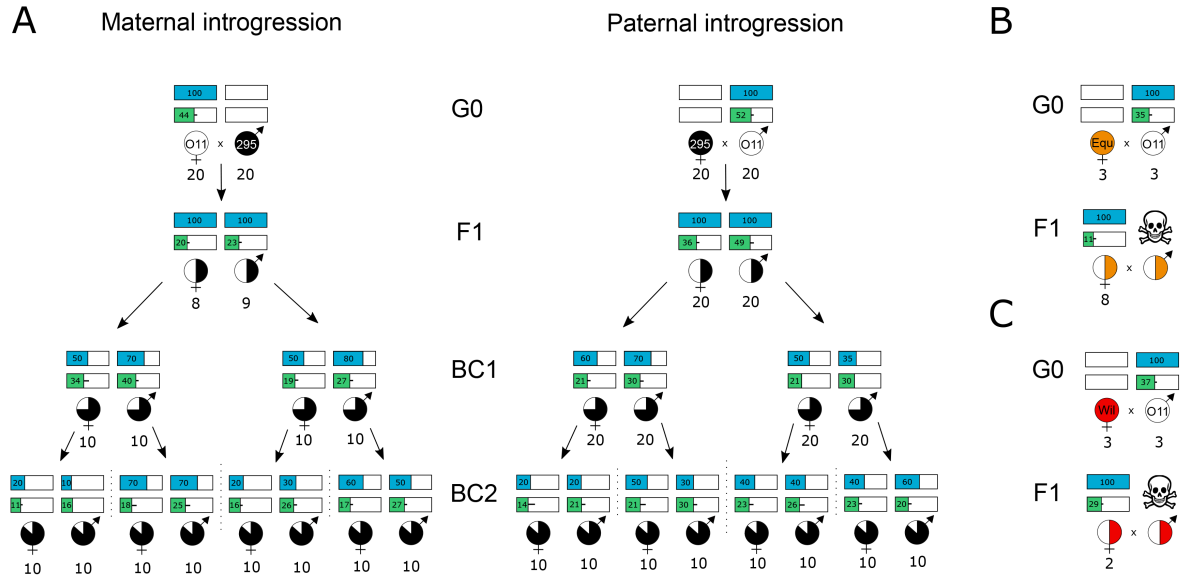
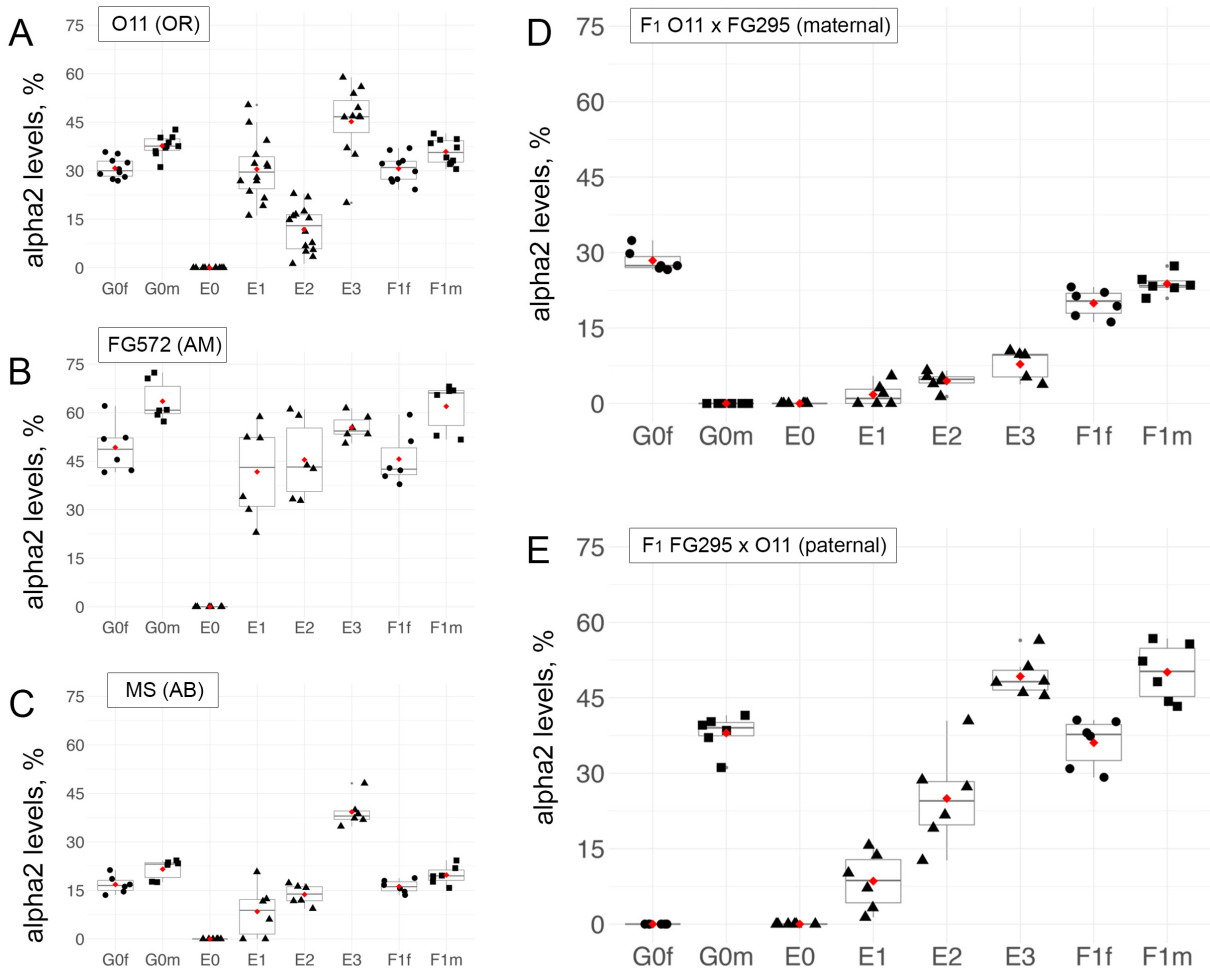


Figure 6. Prevalence and levels of the α2 mitotype introgressed in different genetic backgrounds. (A) Dynamics of α2 prevalence (blue) and levels (green) determined by α-diagnostic *CO1*/*Hin*dIII RFLP in females and males by backcrossing F_1 flies derived from O11 x FG295 (left: maternal introgression; white x black) and FG295 x O11 (right: paternal introgression; black x white) parents to FG295 flies. Arrows in this figure represent a backcross to homoplasmic (β2) FG295 flies. (**B, C)** Dynamics of α2 prevalence (blue) and levels (green) determined by the α2-diagnostic CO2/*Msp*I RFLP in F_1 flies from interspecies crosses of **(B)** *D. equinoxialis* (orange) and **(C)** *D. willistoni* (red) females to heteroplasmic *D. paulistorum* O11 males (white). The skull symbolizes complete absence of F_1 males in both crosses. Lines inside the green bars show standard deviation. Values below sex symbols indicate the number of single flies used in RFLP-PCR analysis and listed in **Table S9**.

α2 dynamics during embryogenesis in heteroplasmic lines and F_1 hybrids uncover their biparental transmission mode

As demonstrated by our crossing experiments, the biparentally inherited α2 mitotype of *D. paulistorum* is highly invasive and maintained at consistently high levels in the adult fly. Since the number of mitochondrial DNA copies per sperm cell is much smaller than in a mature egg [59], and paternally leaked mitochondria are usually both passively diluted and actively destroyed [15], we asked how paternally inherited α2 mitochondria avoid depletion. By using RFLP-PCR, we analyzed α2 levels in unfertilized eggs and embryos from the O11, FG572 and MS lines (**Fig 7A-C**) as well as F_1 embryos from reciprocal crosses between O11 and FG295 flies (**Fig 7D, E**). The α2 levels at different stages of embryogenesis were compared to those in unfertilized eggs (E0) and parental flies (G_0f - females, G_0m - males). Strikingly, none of the five lines analyzed showed traces of α2 in unfertilized eggs. The finding is not

515 surprising for the F_1 flies from FG295 x O11 cross (**Fig 7E**), where the eggs come from
516 homoplasmic FG295 mothers with only the β2 mitotype, but is unexpected for mothers of the
517 four heteroplasmic lines (**Fig 7A-D**) that carry high α2 levels (**Table 1**). We infer that α2 levels
518 are very low in unfertilized eggs of heteroplasmic mothers and below the detection level of our
519 RFLP-PCR method. Immediately after fertilization (E1; 0-30 minutes after egg laying - AEL),
520 however, α2 reached relatively high levels (similar or close to parental) in F_1 embryos of all of
521 the lines except for the ones obtained from the maternal O11 x FG295 cross (**Fig 7D**). For
522 O11 and FG572 E1 embryos (**Fig 7A, B**) median α2 levels were not significantly different from
523 the levels of either parent (p>0.05; here and further on ANOVA with post-hoc Tukey HSD test),
524 except for FG572 males (p<0.01; Supplemental data of **Fig 7**). Median α2 levels of E1
525 embryos from MS, FG295 x O11 and O11 x FG295 (**Fig 7C-E**) were significantly lower than
526 in parents (p<0.05). In mid-embryogenesis (E2; 3-4 hours AEL) α2 levels of O11 were only
527 significantly decreased compared to E1 (p<0.001; **Fig 7A**), whereas in the four other lines (**Fig**
528 **7B-E**) α2 levels continued to increase (statistically non-significant in FG572, MS embryos and
529 F_1 of O11 x FG295, p>0.05; but significant in F_1 of FG295 x O11 crosses, p<0.001). In late
530 embryogenesis (E3; 18-20 hours AEL) all lines showed a significant increase in α2 levels
531 compared to E2 (p<0.001) except for FG572 and F_1 embryos from the maternal O11 x FG295
532 cross shown in **Fig 7B** and **D** (p>0.05). Only in F_1 embryos from the maternal O11 x FG295
533 cross α2 levels at E3 were significantly lower in comparison to parents (9.7%; p<0.001). In all
534 other cases the level of α2 in E3 embryos reached similar or even significantly higher levels
535 compared to their respective heteroplasmic parents (p<0.001 for G_0f in O11 and MS as well
536 as G_0m in MS; p<0.05 for FG295 x O11; p>0.05 for G_0f in FG572, FG295 x O11 and G_0m in
537 O11 and FG572).
538 Additionally, we compared α2 levels of embryos to the levels in emerged flies (F_1f and
539 F_1m, **Fig 7**). There were no significant differences in α2 levels between G_0 and F_1 flies of the
540 same sex except for F_1 flies from the intra-semispecies crosses O11 x FG295 and FG295 x
541 O11 (p<0.01). In comparison between F_1 flies and embryos of heteroplasmic lines we found
542 similar statistically significant differences as between embryos and G_0 flies (see Supplemental
543 data of **Fig 7**). F_1 flies from both O11 x FG295 and FG295 x O11 crosses (**Fig 7D, E**) had
544 significantly higher α2 levels than at any embryonic stages (p<0.05), except in late embryos
545 (E3) from the paternal FG295 x O11 cross with no significant difference to F_1 males (p=0.99),
546 but significantly higher α2 levels than F_1 females (p=0.002). Strikingly, a comparison of the
547 maternal (**Fig 7D**) and paternal (**Fig 7E**) α2 dynamics between embryonic stages (E1-E3) and
548 F_1 adult flies (**Fig 7D, E**) uncovered significant differences in α2 levels depending on the mode
549 of transmission (p<0.001 for all embryonic stages and F_1 flies). Hence during *D. paulistorum*
550 embryogenesis, paternally introgressed α2 mitochondria are replicating faster and possibly
551 earlier than α2 that were transmitted by the female.



552

553 **Fig 7. Relative α2 levels during *D. paulistorum* embryogenesis determined by**
554 ***CO1*/*Hin*dIII (A, B, D, E) and CO2/*Msp*I (C) RFLP-PCR**. Scatter dot plots combined with box
555 plots show levels of α2 (%) in the heteroplasmic lines O11 **(A)**, FG572 **(B)**, and MS **(C)** and
556 α2 levels in F_1 hybrids from maternal **(D)** and paternal **(E)** introgressions obtained from
557 reciprocal crosses between parental heteroplasmic O11 and homoplasmic FG295 flies.
558 Dynamics of α2 levels are presented in adult parental females (G_0f) and males (G_0m),
559 unfertilized eggs (E0), embryos of 0-30 min (E1), 3-4 hrs (E2), and 18-20 hrs (E3) of
560 development at 25°C and F_1 adult females (F_1f) and males (F_1m). Each dot represents the α2
561 level in percent determined from a pool of 20 staged embryos collected for each sample.
562 Females are shown as circles, males as squares and embryos as triangles. Mean values for
563 every stage and sex are depicted with red rhombus.
564
565 Collectively, these data strongly suggest that α2 arrives in very low copy numbers from both
566 sexes to the mature egg and, in order to avoid out-dilution by the primary mitotype, starts
567 replicating immediately after fertilization. This strategy works regardless of its transmission
568 mode and thereby ensures that α2 already reaches high levels in mature embryos (E3),
569 equivalent to levels of the adult fly. As the strict maternally-inherited primary mitochondria of
570 *D. melanogaster* and other animals are quiescent and do not replicate during early
571 embryogenesis [13, 60], this early replication behavior is unique for α2 and we hence name it
572 "Replication precox" (see Discussion).
573

574 **Genomic features unique to the α2 mitotype**

575 The unusual properties of the α2 mitochondria prompted us to search for unique genomic
576 features that distinguish this secondary mitotype from the other *D. paulistorum* mitochondria
577 (**Table 2, 3**). However, only three non-synonymous substitutions were found to be exclusive
578 to the α2 mitotype (**Table 2**).

579

580 **Table 2. Mutations unique to the α2 clade among protein-coding genes of the sampled**
581 ***D. paulistorum* lines**. Three non-synonymous substitutions are unique to α2. Among these,
582 only the H -> N substitution in COIII results in a change in amino acid property. Amino acid
583 and nucleotide changes are presented from other mitotypes to α2. Nr = number.

Gene	Nr non-synonymous	Nr synonymous	Amino-acid change	Chemical properties
ATP6	0	3		
ATP8	0	1		
COI	0	5		
COII	0	1		
COIII	1	0	H -> N	Positive charge to neutral
CYTB	0	2		
ND1	0	2		
NADH2	0	3		
ND3	1	0	V -> M	No change
ND4	1	3	V -> I	No change
ND4L	0	0		
ND5	0	4		
ND6	0	4		

584

585 One of the substitutions is found in *ND3* and causes a replacement of Valine with Methionine
586 (aa position 18 in *D. paulistorum*, 21 in humans) and another substitution in *ND4* causes a
587 replacement of Valine with Isoleucine (aa position 379 in *D. paulistorum*, 391 in humans). Both
588 of these replacements are between chemically equivalent amino acids and likely have little
589 functional impact on the proteins. However, the substitution in *CO3* causes the replacement
590 of a positively charged Histidine by a neutral Asparagine in α2 (aa position 7 in *D. paulistorum*,
591 6 in humans) and is predicted to have medium functional impact according to the
592 MutationAssessor server (http://mutationassessor.org/r3/). An inspection of multi-species
593 alignments in Uniprot and Pfam revealed that Asparagine is rarely found at this position of the
594 *CO3* protein in other animals. The only organisms in the database with Asparagine in the
595 same position are a few species of mites, representatives of some invertebrate marine phyla,
596 and a wide variety of mollusks. Interestingly, among the mollusk sequences, many are from
597 the F mitotype of heteroplasmic unionid mussels with doubly uniparental inheritance (DUI).
598 Additionally, the selfish α2 mitotype differs from the primary mitotypes of *D. paulistorum* by
599 four A to G transitions in the large rRNA gene and a T to A transversion in tRNA-Ala and a 3
600 bp insertion in tRNA-Leu2 (**Table 3**), both of which are located in the T-loop.

601

602 **Table 3. Mutations unique to the α2 subclade among non-protein-coding genes in the**
603 **sampled *D. paulistorum* lines.** Substitutions are found in the large rRNA and tRNA-Ala while
604 an insertion occurs in the tRNA-Leu2. In both tRNAs, the mutations are located in the T-loop.

Gene	Number of mutations	Nucleotide changes
lrRNA	4	A -> G (4x)
tRNA-Ala	1	T -> A
tRNA-Leu2	1	- (β) / AT (α1) -> ATATT

605

606 Since the nuclear-encoded protein Elongation factor Tu (EFTu) is known to interact specifically
607 with the tRNA T-loop during translation [61, 62], we took a closer look at this protein in *D.*
608 *paulistorum*. Two divergent copies of EFTu are encoded in the nuclear genome of *D.*
609 *melanogaster*, *mEFTu-1* and *mEFTu-2*. The two genes have largely sex-specific expression
610 in adults [63] and the proteins differ somewhat in binding specificities to tRNAs with and
611 without T- and D-arms [64]. Using our previously assembled transcriptomes of A28, C2 and
612 O11 [50] we identified orthologs of the two *D. melanogaster mEFTu* genes in *D. paulistorum*.
613 An alignment of the two proteins together with orthologous proteins from all *Drosophila*
614 species found in Flybase (release 2019_04) showed that mEFTu-2, the gene with testis-
615 specific expression, has unique amino acids in two positions in *D. paulistorum*. Interestingly,
616 both positions are close to the C-terminal end of the protein, which is the region that interacts
617 with the T-stem of the tRNA [64], suggesting a potential mito-nuclear coadaptation. However,
618 since only one of the three sequenced *D. paulistorum* lines currently carries the α2 mitotype,
619 and we have no data to say whether the other two, A28 and C2, can carry or have ever carried
620 the α2 mitotype, no firm conclusions can be drawn.
621

622 **The evolutionary history of mitochondria in *D. paulistorum* and the *willistoni***
623 **subgroup**

624 Well-supported phylogenies can provide useful insights into the evolutionary history of genetic
625 elements. Although our whole-genome mitochondrial phylogeny showed very high support in
626 almost all internal nodes, the node defining the relationship between α, β and the clade
627 containing *D. willistoni*, *D. insularis* and *D. tropicalis* (hereafter referred to as the willistoni
628 clade) has low support (**Fig 2B**). Hence, in order to confirm the topology of our mitochondrial
629 phylogeny, we investigated the cause of the poorly supported node by testing whether
630 recombination between clades or bias in nucleotide composition could be affecting the
631 phylogenetic signal.

632 *Nucleotide composition creates conflicting phylogenetic signals between the α, β and the*
633 *willistoni mitochondrial clades*

634 We used several methods to search for signs of recombination between the clades. First, we
635 created single gene trees for the 13 mitochondrial protein coding genes and tested for conflicts

636 between the resulting topologies. For eleven of the 13 trees a definite topology could not be
637 determined as the bootstrap support was less than 80% on the node defining the relationships
638 of the α, β and willistoni clades. The remaining two trees had different topologies, as the *ND2*
639 gene featured the α clade as sister either to the willistoni clade (BS = 81%) and the *ND5* gene
640 placed α as sister to the β clade (*ND5* gene, BS = 90%). Second, we ran tests for intragenic
641 recombination on all protein coding genes. Only two genes, *CO2* and *ND3*, were detected with
642 marginally significant signals (p<0.05) using Phipack [65]. Finally, we estimated the
643 recombination over mutation rate along the whole mitochondrial genome to be low (0.01).
644 These results indicate that the recombination rate is low and that recombination signals are
645 weak and inconsistent. Thus, even if some recombination occurred between mitochondria, it
646 is unlikely to be the cause of the low support on the internal node of the whole-genome
647 mitochondrial tree.

648 Nucleotide composition bias and codon usage can also influence phylogenies and are
649 particularly likely to affect the more neutral third codon position. Hence, we built separate trees
650 using either only the third or a combination of the first and second codon positions of the
651 protein coding genes. Interestingly, we observed significantly increased support for α as sister
652 to the willistoni clade when the third codon position was removed (**Fig 2B**). In contrast, the
653 tree based only on the third codon position showed high (Bayesian posterior probability 0.99)
654 or fairly good (ML bootstrap 88%) support for the α and β clades being sisters (**Fig S4**). The
655 conflicting phylogenetic signal between the two first codon positions and the third codon
656 position suggests that nucleotide composition bias and perhaps codon usage are indeed the
657 cause of low support on the node defining the relationship between the α, β and willistoni
658 clades in the whole genome mitochondrial tree.

659 Since the third codon position is more likely to be affected by such biases, we conclude
660 that the topology supported by the first and second codon positions of protein coding genes
661 **(Fig 2B)** is most likely the accurate representation of the evolutionary relationships between
662 mitochondrial clades. Thus, *D. paulistorum* mitochondria are polyphyletic, and α is sister to
663 the willistoni rather than to the β clade. The resulting phylogeny **(Fig 2B)** implies an α-like
664 mitochondrial ancestor in *D. paulistorum,* and suggests that β was most likely acquired from
665 a so far unidentified lineage within the *willistoni* subgroup.

666 *Mitochondrial and nuclear phylogenies show a complex evolutionary history of mitochondria*
667 *in the willistoni subgroup*

668 A comparison between nuclear and mitochondrial phylogenies shows that neither mitotype
669 relationship nor presence of heteroplasmy follow the topology of the nuclear phylogeny.
670 Mitotypes are shared by relatively distantly related lines belonging to different semispecies,
671 as in the case of α1 in C2 (CA) and MS (AB) and β2 in O11 (OR), L1 (IN) and SM (TR),

672 whereas the primary β1 mitotype is exclusively found in AM semispecies (**Fig 2B**).
673 Heteroplasmic lines are also spread across the nuclear phylogeny, and the mitotype that
674 accompanies α2 in heteroplasmy varies according to what semispecies the line belongs. This
675 pattern suggests that the mitochondria of *D. paulistorum* were not only vertically transmitted
676 but have also been introgressed and lost multiple times during evolution. Further introgression
677 events are indicated by the positioning of *D. paulistorum* and *D. equinoxialis* in relation to other
678 species of the willistoni subgroup on either tree. In the nuclear phylogeny, *D. paulistorum* and
679 *D. equinoxialis* are the most derived species in the willistoni group, but the mitochondrial
680 phylogeny places the α and β clades in a basal position with regards to the willistoni clade
681 (**Fig 2A, B)**.

682 *NUMTs provide insights into the evolutionary history of mitochondria in the willistoni subgroup*

683 Insertions of mitochondrial DNA in the nuclear genome (NUMTs) can provide valuable
684 information about mitochondrial evolution. Using Illumina reads we generated whole genome
685 assemblies for all willistoni subgroup lines (**Fig 2A**) and searched for contigs containing
686 sequences with at least 90% identity to our mitochondrial genomes. Potential NUMTs were
687 identified based on (*i*) coverage similar to that of nuclear genes, (*ii*) low identity towards the α
688 and β genomes, and (*iii*) presence of mixed nuclear-mitochondrial sequences (see Methods).
689 Sequences classified as NUMTs were found in assemblies of all fly lines (**Tables S3, S8**).
690 Interestingly, eleven of the 13 *D. paulistorum* lines, including all lines carrying only β
691 mitochondria, shared a putative NUMT that spans a genomic region containing the *ND2* and
692 *CO1* genes. Contigs containing this sequence had similar coverage to nuclear genes in all
693 lines except FG295 and L1, where the NUMT had lower coverage than the nuclear average,
694 and FG572, in which the NUMT coverage was slightly higher than the nuclear average. In the
695 L1 and SM lines, the contigs also included non-mitochondrial sequences which confirm their
696 nuclear location (**Table S3**, **S10**). A phylogenetic analysis of this genomic region suggested
697 that this putative NUMT forms a clade together with the α mitotype and the mitochondria of
698 other willistoni subgroup species (**Fig 8**).
699

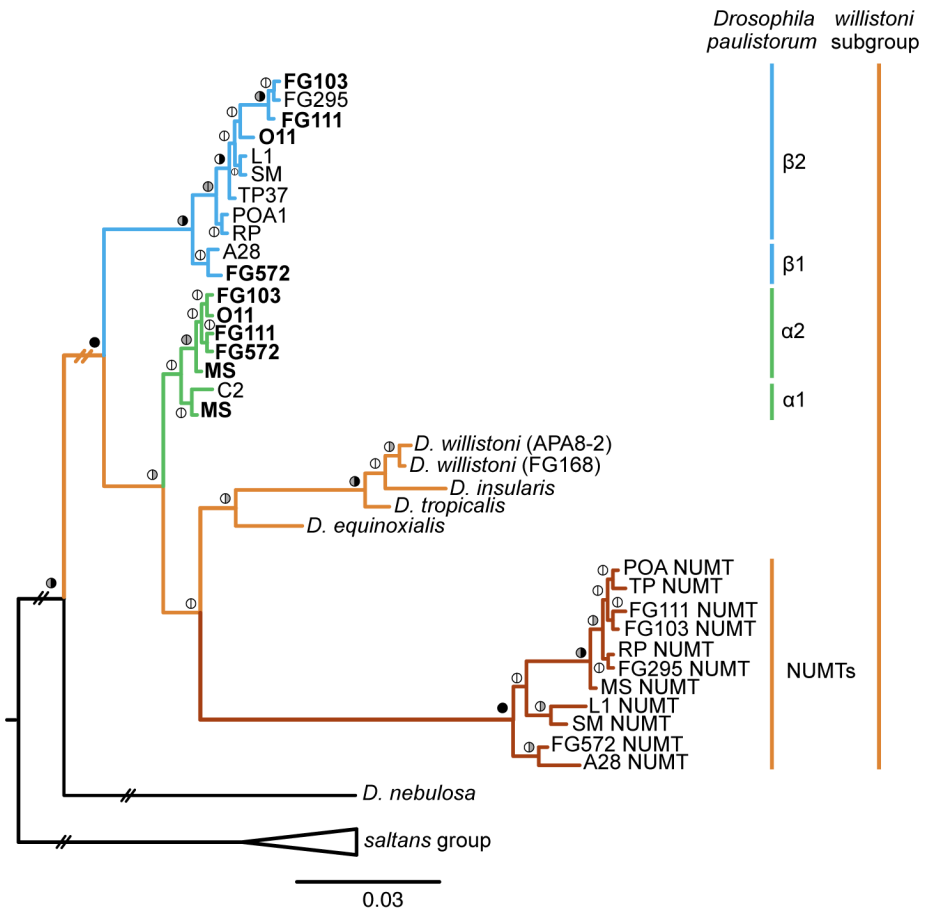


Fig 8. Phylogeny of the mitochondrial region spanning the *ND2-CO1* genes in the willistoni subgroup. NUMT sequences recovered from genomic assemblies of *D. paulistorum* lines branch together with the α and the willistoni clades. Fly lines in bold are heteroplasmic. The same topologies are obtained by maximum likelihood (GTRGAMMA model) and Bayesian analyses. Colored circles next to the nodes indicate ML bootstrap support (left half) and Bayesian posterior probability (right half). Black corresponds to 99-100% bootstrap support or 0.99-1.0 posterior probability, grey represents 70-98% bootstrap support or 0.7-0.98 posterior probability, white indicates support below 70%. Branches leading to the outgroups and marked by two transverse lines were shortened and don't follow the presented scale.

The presence of the sequence in nearly all *D. paulistorum* lines together with the nuclear-like topology suggest that this NUMT stems from a single nuclear insertion that occurred prior to the diversification of *D. paulistorum* semispecies. The ancient origin together with the phylogenetic position of this NUMT close to α and the willistoni clades further support the hypothesis of an α-like ancestral mitotype of *D. paulistorum*.

Finally, we observed that the genome assembly of *D. equinoxialis* contains several β–related contigs, which were not found in species of the willistoni clade (**Tables S3, S8**). The coverage of these contigs is lower than the nuclear average for the line and they do not contain matches to non-mitochondrial sequences. Hence, while these *D. equinoxialis* sequences might stem from NUMTs, we cannot fully exclude that they represent low-level contamination. Nevertheless, it is interesting to note that the presence of β sequences in *D. equinoxialis* might

723 suggest that the last common ancestor of *D. paulistorum* and *D. equinoxialis* carried a β-like
724 mitochondrion.
725

726 ## DISCUSSION

727 We report on a unique system of stable mitochondrial heteroplasmy in the neotropical *D.*
728 *paulistorum* species complex that might exist due to biparental transmission. Using
729 sequencing, RFLP-PCR and bioinformatic analyses we discovered high-level heteroplasmy in
730 41% of the tested fly lines including representatives from three of the six *Drosophila*
731 *paulistorum* semispecies (Andean-Brazilian, Amazonian and Orinocan). However, given the
732 limited number of lines currently available from the Centro-American, Interior and Transitional
733 semispecies, it is possible that heteroplasmy may be even more broadly distributed among
734 the species. We also found that heteroplasmy is fixed with balanced levels of each mitotype
735 in individuals of three long-term laboratory lines (O11, MS and Yellow), but not in the more
736 recently collected lines, in which α2 levels are also tightly balanced but not fixed (**Table 1**).
737 These results suggest that a line has to be genetically homogeneous for heteroplasmy to be
738 fixed and that levels are controlled by the nuclear genetic background. In the course of our
739 current study we observed that the Orinocan FG103 line collected in 2014 lost the secondary
740 mitotype within the first two years in the lab, suggesting that long-term maintenance of fly lines
741 under lab conditions might also contribute to an underestimation of prevalence. Further
742 sampling will be necessary to determine the prevalence of heteroplasmy in natural
743 populations.
744

745 **Function of the α2 mitotype in *D. paulistorum***

746 Using phylogenetic analysis, we found that the mitochondria of *D. paulistorum* are polyphyletic
747 and form two phylogenetically distinct clades - α and β - each further divided into two
748 subclades, 1 and 2. Heteroplasmy is always associated with the α2 mitotype, and this mitotype
749 is in turn never observed in homoplasmy. Such a pattern may occur because α2 alone does
750 not perform all of the functions required for sustaining a fly, or perhaps because homoplasmic
751 α2 lines are rare and difficult to sample in the wild, or both. Although systematic field sampling
752 would be necessary to determine which hypothesis is correct, we found additional evidence
753 suggesting that α2 might not provide full mitochondrial functionality. Mitochondria are usually
754 enriched in energy-demanding tissues [66, 67], and by studying the heteroplasmic O11 line
755 we found that only the β2 mitotype follows this pattern. The α2 mitotype, on the other hand,
756 has unchanging titers across fly tissues. This suggests that β2 is the mitotype that provides
757 energy via oxidative phosphorylation, while α2 does not respond to the energy demands of
758 the host. Despite this, the genes encoded on the α2 genome appear to be evolving under
759 purifying selection, as indicated by low dN/dS ratios and the absence of pseudogenes. In

760 addition, very few genomic differences are seen between α2 and the closely related α1
761 mitotype (0,67% divergence), which since it is the sole mitotype found in two Centro-American
762 *D. paulistorum* lines (C1 and Yellow) must be fully functional. The low sequence divergence
763 between α1 and α2 strongly suggests that the transition from a primary α-like ancestor to a
764 selfish mitotype took place relatively recently. Considering these facts, we surmise that α2 is
765 likely functional based on its genome but that its steady titer throughout tissues and absence
766 in homoplasmy suggest that it does not provide sufficient energy to sustain a fly, at least under
767 the standard laboratory conditions of our experiments.
768
769 **Biparental inheritance of the α2 mitotype in *D. paulistorum***
770 The question remains as to how and why α2 is maintained in *D. paulistorum* flies. It may serve
771 a yet undefined essential or condition-specific function for the fly, or alternatively it may be
772 cheating the system and perpetuate selfishly while providing no or few benefits to the host.
773 One possible way of accomplishing the latter is for the mitotype to increase its own vertical
774 transmission, for example through biparental inheritance.
775 Using RFLP-PCR and discriminant qPCR, we detected high level paternal
776 transmission of the α2 mitotype in offspring from both intra- and inter-semispecies crosses of
777 *D. paulistorum* (**Fig 4, 5 and 6A, B**), and even in offspring from crosses between *D.*
778 *paulistorum* and two closely related species of the willistoni subgroup (**Fig 6C**). None of the
779 other mitotypes found in *D. paulistorum* were paternally transmitted in high levels. Generally,
780 active pre- and post-fertilization recognition and elimination mechanisms prevent paternal
781 transmission of mitochondria in animals (recently reviewed in [15]). In *D. melanogaster,* the
782 mechanism is not yet fully understood, but two genes, *EndoG* and *tamas,* are known to be
783 involved in eliminating mitochondrial DNA from developing spermatids [13, 14, 68]. After
784 fertilization, paternally leaked mitochondria are eliminated by endolytic and autophagic
785 degradation systems [69] while the rare paternal mitochondria that escape are passively
786 outcompeted by the larger number of maternally transmitted mitochondria already present in
787 the egg [70]. In order for a mitochondrion to be transmitted paternally and – even more
788 importantly – to stably persist in adult flies in large numbers, at least one of these mechanisms
789 need to be bypassed. We did not specifically determine if the α2 mitotype evades the active
790 mechanisms that eliminate paternal mitochondrial DNA (reviewed in [15]), but the observation
791 that none of the other mitotypes of *D. paulistorum* were paternally transmitted in large numbers
792 (**Table S7**) implies that there is no general loss of such active defense functions in *D.*
793 *paulistorum*. The spurious low level (~0.1-0.3%) paternal leakage of β2 in some hybrid crosses
794 could be a result of a partial breakdown of such mechanisms due to the mixed nuclear hybrid
795 background ([23] and references therein). However, low levels of paternally transmitted β2 is
796 also in line with previous reports of paternal leakage within *Drosophila* species [26, 29, 30],

797 indicating that similar levels of leakage can occur even without interspecies hybridization and
798 breakdown of defenses. Thus, the mechanism for paternal transmission is specific to the
799 invasive α2 mitotype.

800 In *D. melanogaster* and *D. simulans,* the spermatozoon is estimated to carry
801 approximately 20 copies of mitochondrial DNA [59], which is five to six orders of magnitude
802 less than the number of copies found in an egg [59]. We expected that similarly small numbers
803 of the α2 mitochondrion are present in *D. paulistorum* sperm and at the onset of embryo
804 development when α2 is transmitted by the male. However, using relative RFLP-PCR, we
805 found that rapid and premature replication in early embryos resulted in α2 levels that were
806 similar to those of adult flies just a few hours after fecundation (**Fig 7**). Previous studies of *D.*
807 *melanogaster* showed that maternally transmitted mitochondria are quiescent and do not
808 replicate for up to 10 hours after fertilization [13, 60], which seems to be an evolutionary
809 conserved state (reviewed in [71]). Hence, the premature and unique temporal replication of
810 the α2 mitotype, that we name "Replication precox", might allow this mitotype to escape
811 passive dilution by the still dormant primary mitotype and thereby guarantee its presence in
812 high levels in adult flies. We also found that the α2 mitotype level increased earlier and faster
813 in hybrid embryos when it was transmitted paternally rather than maternally (**Fig 7D, E**). This
814 could be due to more α2 mitochondria being deposited by the sperm than by the egg, or to a
815 higher rate or earlier onset of α2 replication when it is paternally inherited. Since we found that
816 O11 males have significantly higher levels of α2 than females, our data suggest that the former
817 hypothesis is possibly more likely. One tempting mechanistic hypothesis for the Replication
818 precox phenotype is that the nuclear encoded mitochondrial replication machinery is
819 preferentially and prematurely recruited to α2 in the early embryo. The elucidation of the
820 mechanism for diagnostic recognition and selective replication of α2 is currently underway in
821 our laboratory.

822 **Variability in mitochondrial titer and levels between sexes**

823 We found that α2 titers were consistently and significantly higher in males than in females.
824 Since α2 does not seem to be a fully functional mitotype, the higher α2 titer in males cannot
825 be interpreted purely based on different energy needs between sexes. So why would males
826 have a higher titer of α2 mitochondria than females? One possibility is that high titer in males
827 has evolved specifically to facilitate paternal transmission of the invasive α2 mitotype, since a
828 higher titer increases the chance of transmission. Alternatively, higher male titer and the
829 Replication precox phenotype of α2 could have co-evolved in order to compensate for a
830 reduced functionality of the maternally transmitted primary mitotype in males. The mother's
831 curse hypothesis suggests that DNA of strictly maternally inherited mitochondria co-evolve

832 with females and might therefore accumulate mutations that are harmful to male functions
833 [72]. If such mutations have accumulated in the maternally transmitted primary mitochondria
834 of *D. paulistorum*, the biparentally transmitted α2 mitotype might have co-evolved with males
835 and express a still undetermined function that is more important to males than females. In the
836 O11 line, higher relative titer of α2 compared to the primary mitotype β2 are found in heads
837 and gonads (**Fig 3B**), suggesting that perhaps behavior and fertility might be important further
838 areas of investigation for α2 specific functions in males.

839 In addition, our data suggest that the total mitochondrial titer is significantly affected by
840 the presence of α2, as this secondary mitotype appears in addition to the primary
841 mitochondrial copies in a cell. A similar quantitative effect on mitochondrial copy numbers was
842 previously observed in heteroplasmic *D. subobscura* flies carrying a selfish mitotype with a
843 large deletion that results in a replicative advantage [73].

844 Our crossing data also suggests that the nuclear genetic background influences the
845 total mitochondrial titer, particularly in males. This is indicated by a significant reduction in
846 overall mitochondrial titer between pure O11 heteroplasmic males and heteroplasmic hybrid
847 offspring of both intra- and interspecific crosses, but not between pure O11 heteroplasmic
848 females and hybrid offspring. This observation is in line with long-term co-evolution between
849 the heteroplasmic cytoplasm and the nuclear genome in males. In this context it is interesting
850 to note that two of the genomic differences that we found in the α2 mitochondrial genome are
851 located in the T-loops of tRNAs and the nuclear encoded gene *mEFTU-2* that is specifically
852 expressed in testis of *D. melanogaster* has mutations in a region where the protein is predicted
853 to interacts with the tRNA T-loop [63, 64]. Hence, if assuming a similar male-specific
854 expression of *mEFTU-2* in *D. paulistorum*, this observation might strengthen the idea of a co-
855 adaptation between the α2 mitotype and male functions.

856 **Evolutionary history of the α and β mitotypes in the neotropical willistoni group**

857 The ancestrality of α-like mitochondria in *D. paulistorum* is strongly supported by our
858 phylogenetic analysis after we accounted for the nucleotide composition bias in our
859 mitochondrial phylogeny, since the α clade is clearly sister to the other willistoni group species.
860 Furthermore, the presence of an α-related NUMT, which is shared by most sequenced *D.*
861 *paulistorum* lines (**Table S10**) and shows a topology that is similar to our nuclear tree (**Fig 8**),
862 indicates that this sequence was transferred from an α-like ancestral mitochondrion to the
863 nuclear genome before semispecies diversification.

864 Since the β mitotype does not share an immediate common ancestor with α or any
865 other lineage in our phylogeny, its presence in *D. paulistorum* is likely the result of a "ghost"
866 introgression from an unsampled or possibly even extinct species [55-57]. The phylogenetic
867 position of β suggests an origin within the willistoni group but outside the willistoni subgroup.

868 Thus, possible donors include species from the other two subgroups within the willistoni group, the
869 bocainensis and the alagitans. Unfortunately, sequence data is not available for all species in
870 the *bocainensis* subgroup, but *D. nebulosa,* which is included in our phylogeny, branches
871 basally to β. A similar position was observed for three other representatives of the bocainensis
872 subgroup, *D. capricorni, D. fumipennis* and *D. sucinea* in previously published single gene
873 trees [54]. No sequence data is available for any alagitans species. Alternatively, the β
874 mitotype may be the genomic remnant of an extinct species of the willistoni group which once
875 hybridized with *D. paulistorum*. Cases of introgressions followed by extinction of the donor
876 species have been reported in a variety of organisms and are often associated with deep
877 mitochondrial divergence in clades with otherwise high nuclear similarity [57, 74]. To our
878 knowledge, the *D. paulistorum* species complex is the first reported case of stable
879 heteroplasmy originating from mitochondrial introgression.

880 Our analyses allow us to further infer that the introgression of β likely happened before
881 the diversification of the semispecies in *D. paulistorum*. The presence of the β mitotype in 11
882 of 17 sampled lines from four different semispecies of *D. paulistorum* makes it more
883 parsimonious that the mitotype was inherited once from a common ancestor rather than
884 acquired multiple times. The ancestral introgression of β is further suggested by the observed
885 convergence in third codon position between the α and β mitotypes (**Fig S4**). Regardless of
886 whether the underlying mechanism of convergence is exposure of the β genome to the
887 mutational bias (amelioration) or the selective pressure (codon usage) of *D. paulistorum*, it
888 likely required significant time to act. A third indication that β introgression predates *D.*
889 *paulistorum* diversification is the presence of potential β-NUMTs in *D. equinoxialis*. Although
890 we could not conclusively determine the nuclear origin of these sequences, their presence
891 might indicate that *D. equinoxialis* at some point carried a β-like mitochondrion. If so, β could
892 have been introgressed already in the common ancestor of *D. paulistorum* and *D. equinoxialis*.

893 A comparison between the nuclear and mitochondrial trees suggests that several other
894 introgression events might have occurred in the willistoni subgroup apart from the acquisition
895 of β. We observed that the α clade branches basally to the willistoni clade even though nuclear
896 data positions *D. paulistorum* as one of the most derived species in the willistoni subgroup.
897 This suggests that perhaps the extant α mitotype does not directly descend from the ancestral
898 mitochondria of *D. paulistorum*, even if the ancestral mitochondrion was also an α-like
899 mitotype. Instead, the phylogenetic placement of all extant α mitochondria indicates that an
900 introgression in the ancestor of *D. paulistorum* and *D. equinoxialis* from an unsampled deep
901 diverging α-carrying lineage within the willlistoni group might have given rise to the current α
902 mitotypes in a similar mode to what we propose for β. One possibility is that the two extant
903 mitotypes were introgressed at the same time, either together or separately.

904 Incongruences between the nuclear and mitochondrial trees also indicate more recent
905 introgressions between *D. paulistorum* semispecies and lines, as mitotype distribution does
906 not follow the nuclear phylogeny. Since *D. paulistorum* is a recent radiation, i.e., a species
907 cluster *in statu nascendi* ([44] and references therein), such pattern could be explained by
908 hybridization in a more recent past, when the pre- and postmating incompatibilities that exist
909 today between semispecies (reviewed in [75], [44], and this study) were still incomplete.
910 Although both F_1 hybrid sexes from our inter-semispecies crosses were sterile, which would
911 have prevented mitochondrial introgression, possibly some rare fertile F_1 female flies could be
912 found with extended tests and/or other line combinations. As discussed in early literature [76],
913 it is possible that sporadic hybridization occurs between less discriminating and partially
914 compatible populations or semispecies, and those could perhaps lead to the serendipitous
915 events of mitochondrial introgression. As shown earlier, the Transitional semispecies is
916 particularly permissive to inter-semispecies crossings and has the potential to bridge borders
917 [77]. Specific lines of other semispecies have also been shown to be less isolated from others,
918 potentially allowing at least some rare cases of genetic leakage and exchange [78]. For the
919 one Transitional line included in our study, SM, we see a strong discrepancy between the
920 nuclear and mitochondrial trees, with the mitochondrial genome nested within the Orinocan
921 mitochondrial clade and the nuclear tree positioning it with Centro-American. Both our
922 phylogenetic analysis and experimental data from crosses suggest that α2 mitochondria are
923 particularly likely to be transferred in crosses between semispecies, and even when crossed
924 with sister species.
925 Another possible driver of mitochondrial introgression after a hybridization event is the
926 maternally inherited endosymbiont *Wolbachia*. *Wolbachia* strains with strong reproductive
927 manipulation phenotypes or which provide enhanced host fitness can spread fast in the
928 population and are known to cause sweeps of the mitotype associated with infected females
929 [79, 80]. *D. paulistorum* is known to carry obligate *Wolbachia* symbionts, but it is not yet known
930 if these cause any reproductive manipulation besides premating isolation [47, 49]. Thus, we
931 currently have no indication that *Wolbachia* has any part in the introgression, past or on-going.

932 **Introgression and heteroplasmy as drivers of the evolution of selfish mitochondrial**
933 **traits**

934 Our inferences on mitochondrial evolution within *D. paulistorum* provide us with a hypothesis
935 about how features specific to α2 may have evolved. We speculate that the introgression of β
936 into an α-carrying *D. paulistorum* likely created competition between mitotypes for a chance
937 to be transferred to the next host generations. If the ancestrally introgressed β mitotype was
938 more efficient at maternal transmission or perhaps just a better energy producer than the
939 ancestral α, selection could have made it the more dominant mitotype in the *D. paulistorum*

940 species complex. Over time, this could have led to a reduction in prevalence and possibly
941 resulted in extinction of the α mitotype from the species. In this scenario, the evolution of
942 biparental transmission and the Replication precox phenotype may have allowed the α2 lineage
943 to persist selfishly next to the primary mitotype. Such traits would perhaps guarantee the
944 transmission of α2 to the next host generation independently of its ability to meet the energetic
945 needs of the host or to compete with the maternal mitotype for presence in the egg.

946 **Can we be sure that α2 is cytoplasmic?**

947 Finally, we are aware of a potential alternative hypothesis which would suggest that α2 is not
948 a cytoplasmic mitotype but might stem from a so-called "mega-NUMT". It was recently shown
949 that mega-NUMTs can mimic heteroplasmy and extremely rare cases of paternal
950 mitochondrial DNA leakage in humans and other vertebrates (recently reviewed in [81]). In
951 this case, mitochondrial DNA-concatemers produced by extrachromosomal rolling circle
952 replication were found as large-sized chromosomal insertions in human autosomes and
953 wrongly assumed to be unusual biparental transmission of an additional mitotype [82].
954 However, in *D. paulistorum*, we reject this alternative hypothesis based on the following
955 reasons: Contrary to mega-NUMTs, which rapidly accumulate degenerative mutations and
956 structural variation, the genome of the α2 mitotype resembles a fully functional mitochondrial
957 DNA in size and content. Furthermore, the α2 genome is highly conserved within and between
958 heteroplasmic *D. paulistorum* semispecies as well as between long-term lab lines and recently
959 collected lines, which suggests that it evolves under purifying and not neutral selection. In
960 addition, mega-NUMTs are cryptic and if detected they are transmitted at very low frequencies
961 and thereby do not follow strict Mendelian inheritance. This is in sharp contrast to α2 that
962 shows almost perfect biparental transmission within a line and upon introgression this selfish
963 mitotype has the capacity for invasive spreading. Finally, our backcross experiments by
964 introducing α2 in a homoplasmic nuclear backgrounds clearly demonstrate the persistance
965 and spreading capacity of this selfish mitotype, which is self-sufficient and hence not
966 associated with any mega-NUMTs.

967 ## CONCLUSION

968

969 In this paper we present *D. paulistorum* as the first example of an animal with high level, stable
970 heteroplasmy, which is derived from introgression and is robustly maintained in both sexes.
971 The system is remarkable for involving a secondary mitotype with a seemingly functional
972 genome, which appears to not respond to the energy demands of the host and instead relies
973 on biparental inheritance and rapid replication in the early embryo as potentially selfish

974 strategies for spreading and persisting. Additionally, its higher titer and tighter co-evolution
975 with the nuclear background in males might hint at a specific male related function.
976 Given its intact putatively functional genome and recently evolved selfish phenotype,
977 the α2 mitotype can potentially be referred to as a sexually transmitted, parasitic infection
978 rather than a primary maternally inherited mitochondrion. Hence, since α2 appears to have
979 assumed a lifestyle with less host control than typical primary mitochondria, we propose to
980 name it "*Spartacus*" in reference to the famous ancient Roman slave, gladiator and then
981 commander who escaped from imperial oppression by rebellion, for at least a short period of
982 his lifetime.
983 Our results contribute to the increasing realization, driven by recent large-scale
984 sequencing efforts, that animal mitochondria often do not follow the dominant model of
985 homoplasmic, maternally inherited organelles, neither on ecological or evolutionary times
986 scales. Sometimes even organisms, which have been studied for decades, such as *D.*
987 *paulistorum,* harbor unexpected complexity.
988

989 # MATERIALS & METHODS

990 **Fly strains, semispecies typing, and *Wolbachia* knockdown**
991 Information regarding the *Drosophila* lines used in this study is summarized in **Table S1**. All
992 flies were kept at 25±1°C on Formula 4-24 *Drosophila* instant food (Carolina, USA) with a 12
993 hr light-dark cycle. Typing of the biological semispecies of the *D. paulistorum* isofemale lines,
994 which were recently collected from French Guiana (FG16, FG103, FG111, FG295 and FG572),
995 was done by analyzing their reproductive compatibility towards reference lab strains from each
996 of the six semispecies (A28 (AM), O11 (OR), C2 (CA), L1 (IN), SM (TR) and MS (AB)**).** Each
997 new line was assigned to the semispecies with which it produced fertile F1 offspring. Crosses
998 were performed in 2-3 biological and three technical replicas (in total 30 crosses in 2-3
999 biological replicas). Each biological replicate consisted of 3-6 females and 3-6 males, which
1000 were kept in the same vial for 3-5 days before transfer into a new vial for generating one
1001 technical replicate. Egg-to-adult development was monitored and information on all crosses
1002 including the number of F_1 flies hatched is summarized in **Table S6**.
1003
1004 *Wolbachia knockdown*
1005 In order to test the potential effect of the *Wolbachia* endosymbiont of *D. paulistorum* [47] on
1006 mitotype persistence and levels, we generated lines with lowered *Wolbachia* infection titer.
1007 *Wolbachia* was knocked down by keeping flies on food containing 0.05% tetracycline [w/v] for
1008 four generations. Flies were repeatedly tested using PCR in order to confirm the absence of
1009 the symbiont. After four generations, knock-down flies were moved to tubes previously

1010 occupied by males of the same line, so that they could feed on feces of flies with wild-type gut
1011 microbiota. This aimed to restore any gut flora affected by the antibiotic and keep the
1012 knockdown and wild-type fly lines as similar as possible with the exception of *Wolbachia*
1013 presence [49].
1014
1015 **Single fly Southern blots**
1016 Single fly Southern blots were performed following the protocol by [83]. In brief, DNA was
1017 extracted from single individuals, digested with the restriction enzyme *Hin*dIII and processed
1018 for electrophoresis on a vertical 0.8% agarose gel. After vacuum-blotting onto a positively
1019 charged nylon membrane, fragments were hybridized with P_{32}-labelled mitochondrial *CO1*
1020 probe derived from a plasmid. Detection was performed by exposing the membranes to X-ray
1021 films at -80 degrees.
1022
1023 **Restriction Fragment Length Polymorphism-PCR (RFLP-PCR)**
1024 Total DNA was extracted from single or multiple flies using the Gentra Puregene Tissue kit
1025 (Qiagen, Germany). Flies were homogenized using a TissueLyser LT (Qiagen) at 50 Hz for
1026 50 sec and DNA was consequently isolated following the manufacturer's protocol. DNA
1027 concentration was determined with a NanoDrop OneC Spectrophotometer (Thermo Scientific).
1028 RFLP-PCR for the mitochondrial Cytochrome c oxidase genes *CO1* and *CO2* was set up in
1029 20 µl reactions containing 1x Promega reaction buffer, 2.5 mM $MgCl_2$, 0.5 µM of each primer
1030 (**Table S11**), 200 µM of each dNTP, and 0.025 U of GoTaq G2 DNA Polymerase. *CO1*/*CO2*
1031 reactions were performed using the following thermal profile: initial denaturation for 2 min at
1032 95°C followed by 30 cycles consisting of 45 sec denaturation at 95°C, 45 sec annealing at the
1033 appropriate primer conditions Tm°C (**Table S11**) and 30 sec extension at 72°C. Final
1034 extension was carried out at 72°C for 10 min. PCR products were consequently used for RFLP
1035 (digest plus undigested control) using the restriction enzymes *Hin*dIII and *Eco*RV-HF for *CO1*,
1036 and *Msp*I for *CO2*. Reactions were performed in 20 µl volume containing 10 or 20 U of enzyme,
1037 1x reaction buffer and 10 µl PCR product. After overnight incubation at 37°C, enzymes were
1038 inactivated by either heat (80°C for 20 min) or by adding EDTA-containing gel loading dye. 10
1039 µl of undigested and digested samples were then run on a 1.5% agarose gel and post-stained
1040 with 10 mg/ml ethidium bromide. Imaging was performed using a Molecular Imager
1041 ChemiDocTM XRS Imaging System and mitochondrial heteroplasmy levels were quantified
1042 using Image Lab 5.2.1 software.
1043
1044 *Quantifying levels of heteroplasmy*
1045 Undigested samples representing the sum of both mitotypes were set as reference band with
1046 relative quantity 1. The relative levels of mitochondria were then quantified by summing up the
1047 two digested fragments plus the undigested fragment. Due to technical differences in band

1048 intensity, however, the sum of all fragments did not result in a relative quantity of 1. Hence the
1049 actual sum value was set to 100% to determine the percentage of mitotype levels. Smaller
1050 fragments with length up to approximately 100 bp were not detectable after ethidium bromide
1051 staining and we hence used the correction factor $Cf = \frac{undigested[bp]}{digested[bp]}$ to estimate their relative
1052 quantities.
1053
1054 **DNA extraction, genome sequencing and assembly**
1055 For each line 20 ovaries were dissected from 3-day old adult females and DNA extraction was
1056 performed using the Gentra Puregene Blood and Tissue kit (Qiagen). DNA samples used for
1057 PacBio sequencing were produced using the protocol described in [84]. Briefly, *Drosophila*
1058 flies were transferred to apple juice agar plates and allowed to oviposit for 2 hours, after which
1059 the eggs were collected, washed and dechorionated in 50% bleach and manually
1060 homogenized using a plastic pestle. Following centrifugation and filtration of the homogenate,
1061 the resulting cell pellet was subjected to whole genome amplification using the Repli-g® midi
1062 kit (Qiagen) after which the DNA was purified using QIAamp® DNA mini kit (Qiagen) according
1063 to the manufacturer's recommendations.
1064
1065 *Sequencing*
1066 *Drosophila paulistorum* lines from the classic six semispecies plus lines from more recent
1067 collections were used for genome sequencing (**Table 1 and S1)**. DNA extracted from ovaries
1068 was used to construct 350 bp fragment TruSeq libraries, multiplexed and sequenced at the
1069 Uppsala SNP and Seq platform in three different runs on an Illumina 2500 HiSeq machine
1070 generating 2x125 bp reads. The Illumina reads were quality filtered and trimmed using
1071 Trimmomatic-0.30 and error corrected using SPAdes-3.5.0. Amplified DNA extracted from
1072 early embryos from three lines (O11, POA-1 and TP37) was used to create 5 kb fragment
1073 SMRTbell libraries. Each library was run using P6-C4 chemistry in one SMRT cell on a RSII
1074 PacBio instrument. PacBio libraries were produced and sequencing was performed at the
1075 Uppsala Genome Center, Uppsala, Sweden.
1076
1077 *Mitochondrial genome assembly*
1078 The two different mitochondrial genomes α and β of the O11 line were generated by mapping
1079 all Illumina reads to the published mitochondrial genome of *D. willistoni* (NCBI accession
1080 number BK006338.1) using BWA-MEM [85] and thereafter extracting all aligned reads and
1081 their mates using SAMtools [86]. The extracted Illumina reads were then subsampled to
1082 achieve an approximate coverage of 100 and assembled together with PacBio filtered
1083 subreads using SPAdes-3.10.1 [87] with multiple different k-mers settings. The β
1084 mitochondrion consistently came out as a single full length contig, so the best assembly was

1085 chosen based on the contiguity of the putative α mitochondrion. From this assembly the contig
1086 representing the β mitochondrion and the contigs with a homogenous coverage likely
1087 representing the α mitochondrion were taken out and used as trusted contigs together with
1088 the Illumina data to generate a better assembly of the α mitochondrion. Finally, the Illumina
1089 and PacBio data were aligned against the resulting contigs using BWA, MEM and SW
1090 respectively. The resulting bam-file was converted to an ace-file that was used for manual
1091 curation of the sequences in Consed [88]. The mitochondrial genomes of all other *Drosophila*
1092 lines were generated by mapping Illumina reads to the two O11 mitochondria with BWA-MEM,
1093 extracting all aligned reads and their mates using SAMtools and assembling the extracted
1094 reads with SPAdes-3.10.1 with multiple different k-mers settings. Since PacBio data were only
1095 generated from the O11, TP37 and POA-1 lines, the assemblies of all other lines were run
1096 using only Illumina reads. For all lines, this assembly procedure generated one contig that
1097 represented a complete (O11) or nearly complete circular mitochondrial genome.
1098 To test for heteroplasmy and to correct possible errors in contigs, the Illumina reads
1099 for each line were mapped back to the contig containing a complete or nearly complete
1100 mitochondrion for that line using BWA-MEM and indels were realigned with the IndelRealigner
1101 from GATK [89]. The resulting bam-file was then used to call variants using Freebayes with a
1102 range of parameters and converted to ace-format for manual curation in Consed. A line was
1103 determined to be heteroplasmic if 1) at least 80 sites (ca 0,5%) had a variant and 2) the
1104 variants had a consistent alternative allele frequency over the whole or a significant part of the
1105 mitochondrial genome. These settings were chosen to avoid calling variant genomes based
1106 on sequence errors and to make sure that only complete mitochondrial genomes (not NUMTs)
1107 were used to decide heteroplasmy status. If a line was seen to be heteroplasmic using these
1108 criteria, two different alternatives were used to resolve the assembly of the second
1109 mitochondrion. 1) The Illumina reads mapping to the O11 α and O11 β mitochondria were split
1110 into two separate data sets and assembled separately, 2) in the case of MS that has two α
1111 mitochondria, variant calling was performed with lofreq [90], variants with a frequency between
1112 8-30% were selected and used to make a new consensus with bcftools and the reads were
1113 mapped to the two variants of the mitochondrial genome. The same procedure was done in
1114 multiple rounds until no more variant sites within the correct frequency were found. The final
1115 bam-file was converted to ace-format and manually inspected in Consed.
1116
1117 **Alignments and phylogenetic analyses**
1118 Datasets of either whole mitochondrial genomes, nuclear markers or mitochondrial genes
1119 were aligned with MAFFT v6.850b (mafft-linsi algorithm) [91] and trimmed with TrimAl
1120 v1.2rev59 [92] using the automated1 for removal of poorly aligned regions. Concatenated sets
1121 of either mitochondrial or nuclear genes were created by combining trimmed alignments of

1122 single genes. Datasets of mitochondrial protein coding genes containing either only the 3rd or
1123 a combination of 1st and 2nd codon positions were created by parsing the alignments with an
1124 in-house script.
1125 Maximum likelihood trees were built with RAxML version 7.2.6 [93] using -f a -m GTRGAMMA
1126 -x 12345 and -# 1000. Bayesian trees were made with Mrbayes version 3.2.6 [94, 95] using
1127 "nst= mixed" for estimating evolutionary models. Analyses were run for 10.000.000
1128 generations with "nruns= 2", "nchains= 4" and "samplefreq= 1000". A burn-in of 25% was
1129 applied. Trees were visualized in FigTree v. 1.4.3 [96] and rooted on the species of the *saltans*
1130 group.
1131
1132 **Identification of synonymous and non-synonymous substitutions**
1133 Synonymous and non-synonymous substitutions in pairwise comparisons between lines of the
1134 α x β and α1 x α2 clades were identified in single gene alignments using codeml (PAML
1135 package v. 4.8) [97]. Values obtained from pairwise comparisons between lines were then
1136 used to estimate an average for the clades. The same method was used for calculating the
1137 number of nonsynonymous substitutions per non-synonymous site (dN), synonymous
1138 substitutions per synonymous site (dS) and the dN/dS ratios. Synonymous and non-
1139 synonymous mutations unique to α1 and α2 were counted through visual inspection of single
1140 gene alignments using Aliview v. 2019 [98].
1141
1142 **Assessing rarity and functional impact of α2-unique mutations**
1143 We used the MutationAssessor server (www.mutationsassessor.org) to estimate the potential
1144 impact of non-synonymous substitutions exclusive to α2 to protein function. Since the server
1145 only analyses human sequences, we used the Uniprot website (www.uniprot.org) to align *D.*
1146 *melanogaster* and human *CO3*, *ND3* and *ND4* amino acid sequences and identify the
1147 positions in the human genes which correspond to the non-synonymous substitution sites
1148 unique to α2. With that information we generated the following input strings which were then
1149 used in the server: COX3_HUMAN H6N, NU3M_HUMAN T21M and NU4M_HUMAN I391V.
1150 We used Uniprot to assess whether the α2-unique non-synonymous mutation in *CO3* was
1151 found in other species. This was done by searching for "COX3" in the UniProtKB database,
1152 aligning all entries of the first results page (250 entries) and visually checking for the presence
1153 of an N residue in the position of interest. The process was repeated for the subsequent result
1154 pages until 2000 entries had been aligned and checked.
1155
1156 **Quantitative Real-Time PCR (qPCR)**
1157 DNA was extracted by homogenizing whole flies in 200µl Chelex solution at 5% concentration
1158 in distilled water. A total of 10µl of proteinase K (10mg/ml) was added per sample and vials

1159 were incubated for 6 hours at 56°C. After digestion, vials were centrifuged at 16,200 x g for 3
1160 minutes, and the supernatant was transferred to a new vial. DNA was stored at 4°C and
1161 freeze-thaw cycles were avoided when possible.
1162 Samples were run in duplicate using SYBR Green reagent on a StepOnePlus™ Real Time
1163 PCR machine (Applied Biosystems). Technical repeats with >1 Ct difference were rerun or
1164 discarded. Each reaction contained 10µl Maxima SYBR Green/ROX qPCR Master Mix (2x),
1165 1µl of forward primer, 1µl of reverse primer, 6µl nuclease-free water and 2µl of the DNA
1166 sample. Cycling conditions were 95°C for 20 seconds, followed by 40 cycles of: 95°C for 3
1167 seconds and 60°C for 30 seconds for rps, and *Wolbachia* primers (**Table S11**). Discriminant
1168 mitochondrial primers cycling conditions were 95°C for 20 seconds, followed by 40 cycles of:
1169 95°C for 3 seconds and 55°C for 30 seconds (**Table S11**). Discriminant primers used linked
1170 base technology to increase specificity (Exiqon). Melt curves and the fly control gene *rps17*
1171 were run on each plate to control for successful single-peak amplification and to control for
1172 DNA quality. All primers had >90% efficiency. For absolute quantification, each 96-well qPCR
1173 plate was analyzed using StepOne Software v2.2.2 (Applied Biosystems) and Ct values were
1174 obtained by comparing each primer sample to a single standard curve of known concentration
1175 and using identical threshold and baseline levels for each primer target across plates.
1176 Standard curves were created by amplifying positive control samples using PCR, calculating
1177 DNA concentrations using QBIT, and then serially diluting the sample 1:10 with distilled water
1178 to create a 5-sample curve comprising known concentrations decreasing from 10pmol/ ml.
1179 Samples with Ct values over 30 were classed as negative, confirmed by our negative controls.
1180 As a result, the detection limit was 50 copies per sample for *rps17*, 115 for *CO3* (SNP 1), 735
1181 for *CO3* (SNP 2) and 1627 for *CO3* (SNP 3). This varied for the FG295 qPCR, where samples
1182 with Ct values over 20 were classed as negative for the *CO3* (SNP 2) primer due to very high
1183 amplification of some DNA segments. This was confirmed by negative flies, but led to a high
1184 threshold of detection of 6150 for *CO3* (SNP 2). DNA concentration was calculated for each
1185 sample by comparing sample Ct values back to the equation of the standard curve. DNA copy
1186 numbers were then calculated by using DNA product length to determine DNA concentration.
1187 To control for fly size and extraction efficiency, copy numbers for each sample were
1188 normalized using the fly housekeeping gene as a control, giving a final value in terms of
1189 mitochondrial genome copies to fly nuclear genome copy ratio.
1190 Since we used three independent experimental approaches to determine levels of
1191 heteroplasmy in *D. paulistorum*, i.e. NGS, RFLP-PCR, and qPCR, we compared the
1192 robustness of these. We looked at the levels of α2 in O11 females (**Fig S5**), and found that all
1193 three techniques determined similar levels, hence confirming the robustness of our
1194 experimental approach.
1195
1196 **Cross-mating and embryo collection**

1197 For mass-mating crosses (pools), virgin males and females from different *D. paulistorum* lines
1198 were obtained by collecting newly emerged flies within 2-3 h intervals to avoid any possible
1199 sibmating. Collected flies were kept separated for 5-7 days to ensure their virgin status.
1200 Crosses were set up in pools using 10-20 females and 20-30 males per each vial and in 2-3
1201 replicates. Flies were mated for one week and then transferred to new vials or used for
1202 consequent embryo collecting. F_1 flies emerging from the first vials were collected for DNA
1203 extraction. Mass-mating A28 x O11 hybrids were generated by mating 10 five-day-old virgin
1204 A28 females to 10 O11 males. F_1 individuals were used for assays. For generating
1205 interspecies hybrids *D. paulistorum* O11 males were mated either with *D. equinoxialis* or *D.*
1206 *willistoni* virgin females in pools of 20 females and 60 males per cross. F_1 flies emerging from
1207 the bottles were collected for DNA extraction. Single fly crosses were generated by collecting
1208 virgin flies every 6-8 hours to avoid sibling mating. Collected flies were kept separated for 5-7
1209 days to ensure their virgin status before use in crossings. Single pairs of male and female flies
1210 were mated for two weeks in fresh vials. Emerging F_1 flies were collected, aged to 8-10 days
1211 and frozen for subsequent DNA extraction. For FG295 x O11 and O11 x FG295 crosses,
1212 single-pair sibling mating were continued for four consequent generations.
1213
1214 *Backcrossing*
1215 For backcrossing experiments, heteroplasmic F1 flies obtained from reciprocal crosses
1216 between O11 and FG295 parents (G0) were crossed in pools to the homoplasmic FG295 line.
1217 Virgin F_1 males and females were obtained from the G0 crosses by collecting newly emerged
1218 flies within 2-3 h intervals to avoid sib mating. Collected flies were kept separated for 5-7 days
1219 to ensure their virgin status. Backcross 1 (BC1) was set up in pools using 5-10 females and
1220 5-10 males per each vial. The first 10 F_1 flies of each sex emerging from the vials were
1221 collected for further backcrossing with FG295 line and the remaining F_1 flies were frozen in
1222 liquid nitrogen for future DNA extraction. Backcross 2 (BC2) was set up similarly to BC1.
1223 Between 10 and 20 flies were analyzed for each backcross.
1224
1225 *Collection of staged embryos*
1226 *D. paulistorum* embryos at early (0-30 min AEL), mid (3-4 hrs AEL) and late (18-20 hrs AEL)
1227 stages of development were collected on homemade medium (agar, molasses, cornmeal and
1228 yeast medium) in plastic Petri dishes (3 x 3 cm) using collection chambers at 25°C. Prior to
1229 collecting embryos, 1-2 weeks old flies were kept in these chambers for 2-3 days to adapt to
1230 the new environment. Embryos were then manually picked with a dissection needle and
1231 transferred immediately to ice-cold lysis buffer (Qiagen). We collected 20 embryos for each
1232 sample with at least five biological replicates. Embryos were immediately processed for DNA
1233 extraction or stored at -20°C for no more than one week.
1234

1235 **Recombination analysis**
1236 We checked for intergenic recombination between lines by analyzing single gene trees built
1237 as described in the "Alignments and phylogenetic analyses" methods section. Tests for
1238 intragenic recombination on single-gene and whole-genome alignments were done using
1239 Geneconv v.1.81 and PhiPack with default parameters. Recombination over mutation rates
1240 were calculated for single-gene and whole-genome alignments using ClonalFrame v.1.2 with
1241 default settings.
1242
1243 **Nuclear markers**
1244 Illumina reads from each fly line were separately assembled with both Megahit v.1.1.2 [99]
1245 and SPAdes v. 3.13.0 using default parameters. We then blasted the *D. willistoni* CDSs of the
1246 selected nuclear markers (**Table S12**) to each assembly and retrieved sequences with an
1247 alignment length of over 200 bp and above 70% identity. The obtained contigs were aligned
1248 to the corresponding *D. willistoni* gene with MAFFT v6.850b. The alignments were visualized
1249 with Aliview v. 2019 and inspected for assembly issues and, if necessary, multiple exons were
1250 joined into a single sequence using Aliviews' "merge" function. For each fly line and marker
1251 gene, results obtained from both assemblies were compared and only the one with highest
1252 quality was used in further analyses.
1253
1254 **Identification of nuclear mitochondrial DNA (NUMTs)**
1255 Illumina reads of each line were separately mapped with BWA-MEM v.0.7.10 to the genome
1256 assemblies generated with Megahit v. 1.1.2. The average per-base coverage of each
1257 assembled contig was estimated with SAMtools mpileup and contigs with average coverage
1258 lower than 2 were excluded from the analysis.
1259 Each genomic assembly was blasted to all previously assembled *D. paulistorum*
1260 mitochondrial genomes. Sequences corresponding to high scoring pairs (HSPs) with at least
1261 200 bp length and 90% similarity to mitochondrial DNA were retrieved. Recovered sequences
1262 with more than 97% identity to either α or β genomes were deemed "conserved" and
1263 tentatively assigned to the clade they were most similar to, while those with 90-97% identity
1264 were considered divergent. Sequences retrieved from each assembly were mapped back to
1265 an α and a β mitochondrial genome (C2, O11 beta) with BWA-MEM and the number of bases
1266 covered by them were counted with Samtools mpileup. This was done in order to determine
1267 how much of the mitochondrial genome was covered by contigs belonging to each category
1268 (α, β, diverged). Genes present in the retrieved sequences were identified by blasting each
1269 sequence to a database containing all genes from the A28 mitochondrial genome (CDS, tRNA
1270 and rRNA). All contigs containing mitochondrial sequences were blasted against NCBI's nt
1271 database for identification of potential matches to non-mitochondrial references.

1272 Sequences retrieved from each genomic assembly were considered NUMTs if the
1273 contigs in which they were found a) also contained matches to non-mitochondrial references;
1274 or b) had average coverage similar (± 30%) to the reference nuclear coverage of its line. The
1275 reference nuclear coverage for each line was calculated as the average coverage of the
1276 contigs containing the genes *CAD/r*, *Beta Spectrin* and *FASN3*. These were identified by
1277 blasting *D. willistoni* CDSs of these genes to each assembly. The reference mitochondrial
1278 coverage of each line was defined as the average per base coverage of the longest contig
1279 which had 100% identity to the line's assembled mitochondrial genome(s).
1280

1281 **Statistics**

1282 Statistical analyses varied depending on the data source and distribution. For the qPCR tissue
1283 distribution of α and β mitotypes between different ages and sexes we used ANOVA on log-
1284 transformed data to increase normality. In the qPCR analysis of A28 x O11 hybrids, we used
1285 ANOVA with post-hoc Tukey tests of significance. Due to low numbers of repeats and power
1286 in the intraspecies FG295 x O11 hybrids, Wilcoxon Rank Sum tests were used for significance
1287 testing. Assumptions and variance were checked before use. These analyses were
1288 completed using R version 3.3.2 [100].
1289 In the RFLP-PCR experiments, the α2-levels in embryos from different *D. paulistorum*
1290 semispecies and crosses at different developmental stages were compared using One-way
1291 ANOVA with post-hoc Tukey HSD tests. Quantifications were made with the help of an online
1292 calculator (www.astatsa.com). The raw data showing levels of α2 in percent were obtained
1293 with Image Lab 5.2.1 software and transformed from percentage to arcsine for normal
1294 distribution before applying the statistics. RFLP-data were statistically analyzed using ANOVA
1295 and Student's t tests.
1296

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1310

1311 **DATA AVAILABILITY**

1312 Raw sequence data used in this project as well as assembled whole mitochondrial genomes
1313 and nuclear markers are available in NCBI under the Bioproject PRJNA643793. Accession
1314 numbers of individual mitochondrial genomes are listed in Table S2.
1315
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