Evolution of Larval Segment Position across 12 *Drosophila* Species

Running title: Evolution of Segmentation across Drosophila

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Abstract

Many developmental traits that are critical to the survival of the organism are also robust. These robust traits are resistant to phenotypic change in the face of variation. This presents a challenge to evolution. In this paper, we asked whether and how a well-established robust trait, *Drosophila* segment patterning, changed over the evolutionary history of the genus. We compared segment position scaled to body length at the first instar larval stage among 12 *Drosophila* species. We found that relative segment position has changed many times between species. Changes were frequent, but primarily small in magnitude. Phylogenetic analysis demonstrated that rates of change in segment position are variable along the Drosophila phylogenetic tree, and that these changes can occur in short evolutionary timescales. Correlation between position shifts of segments decreased as the distance between two segments increased, suggesting local control of segment position. The posterior-most abdominal segment showed the highest magnitude of change on average, had the highest rate of evolution between species, and appeared to be evolving more independently as compared to the rest of the segments. This segment was exceptionally elongated in the cactophilic species in our dataset, raising questions as to whether this change may be adaptive.

Keywords: segment patterning, Drosophila, evolution, robustness, larval stage

Introduction

Many developmental phenotypes are critical to the survival and fitness of the organism. These phenotypes are often observed to be robust, in that they produce a stereotyped outcome despite variation encountered during development (Wagner 2005; Félix and Wagner 2006; Masel and Siegal 2009; Siegal and Leu 2014). The variation experienced in ontogeny can come in a variety of forms, including stochastic, genetic, and environmental variation (Wagner 2005; Félix and Wagner 2006; Masel and Siegal 2009; Siegal and Leu 2014). Some robust phenotypes have specific mechanisms in place to ensure they are produced faithfully (Félix and Wagner 2006; Masel and Siegal 2009; Siegal and Leu 2014; Nijhout et al. 2017). However, these same robust phenotypes do evolve over periods of evolutionary time (Félix et al. 2000; Arthur and Chipman 2005; Lott et al. 2007; 2010; Fowlkes et al. 2011; Félix 2012; Combs and Fraser 2018). This poses a fundamental question: how do robust traits evolve if the phenotypic variation necessary to selection is suppressed?

One such developmental phenotype is segmentation along the head to tail (anterior-posterior) axis. Segmentation is the periodic repetition of anatomical structures. It is a shared feature of three big animal phyla, annelids, arthropods, and chordates (Davis and Patel 1999; Tautz 2004). In Drosophila, the foundation of segmentation is laid during the beginning of embryogenesis (St Johnston and Nüsslein-Volhard 1992), when development is under control of the maternal gene products, before the zygotic genome is activated. Maternal gene products that are located in the anterior and posterior parts of the fertilized egg set up a concentration gradient along

81 the length of the embryo (St Johnston and Nüsslein-Volhard 1992; Surkova et al. 2018).
82 These maternal gene products regulate one another and regulate downstream targets
83 (St Johnston and Nüsslein-Volhard 1992; Surkova et al. 2018). This genetic network of
84 regulators, which has been well-established through decades of critical study (Nüsslein-
85 Volhard and Wieschaus 1980; Kornberg and Tabata 1993; Nasiadka et al. 2002;
86 Nüsslein-Volhard et al. 2008) precisely divide the embryo into progressively smaller
87 subsections over embryonic development, until the correct number of body segments is
88 reached. At the end of the embryonic stage, a first instar larva with a highly organized
89 segmented pattern of differentiated structures is produced.

90 Given the fundamental role of segmentation in Drosophila development, defects
91 in this process can be detrimental (or lethal) to the organism (Wieschaus and
92 Nuesslein-Volhard 2016). Indeed, the lethality of homozygous mutations in
93 segmentation genes was critical to their discovery in mutant screens (Wieschaus and
94 Nuesslein-Volhard 2016). However, studies have also shown that segmentation can
95 proceed correctly, while keeping its precision, in the face of substantial perturbations.
96 For instance, researchers showed that the stochastic variation between embryos in the
97 anterior-posterior gradient of the maternal gene *bicoid* (*bcd*), a master regulator for
98 embryonic patterning in Drosophila, is 2-5x higher than the variation observed in the
99 position of expression domain borders of downstream genes, particularly that of the gap
100 gene *hunchback* (*hb*) (Houchmandzadeh et al. 2002; Gregor et al. 2007; Manu et al.
101 2009b). The mechanism that cancels out the noise in Bcd gradient at the level of *hb*
102 expression is co-repression of *hb* by two other gap proteins, Kruppel and Knirps (Manu

103 et al. 2009b; Perry et al. 2012; Surkova et al. 2013). It has also been shown that in the
104 face of substantial genetic variation, such as mutations in important maternal genes or
105 absence of whole chromosomes, *hb* expression boundary was affected only
106 incrementally, if at all (Houchmandzadeh et al. 2002). Moreover, variation in the location
107 of *hb* expression boundary did not significantly increase (Houchmandzadeh et al. 2002).
108 Other studies have shown that changing Bcd dosage in the embryo resulted in
109 expansion or reduction of the head region as compared to the rest of the body (Driever
110 and Nüsslein-Volhard 1988; Namba et al. 1997). With some limitations, segment
111 patterning was corrected in these genetically perturbed embryos through increased or
112 decreased apoptosis in the enlarged versus reduced body regions, respectively (Namba
113 et al. 1997). We know that segmentation can also proceed precisely in the face of
114 substantial environmental perturbation. When whole (Houchmandzadeh et al. 2002) or
115 two halves (Lucchetta et al. 2005) of *D. melanogaster* embryos were raised at two
116 temperature extremes after fertilization, inducing different developmental rates
117 (Houchmandzadeh et al. 2002; Lucchetta et al. 2005), there were temperature induced
118 changes in Bcd gradient. However, by the gap or pair rule stage of embryonic
119 development, respectively, segments resumed their normal positions with the same
120 level of precision as control embryos (Houchmandzadeh et al. 2002; Lucchetta et al.
121 2005).

122 Drosophila segmentation is a complex trait that has been demonstrated to be
123 highly robust (Driever and Nüsslein-Volhard 1988; Namba et al. 1997;
124 Houchmandzadeh et al. 2002; Lucchetta et al. 2005; Manu et al. 2009b). It is perhaps

125 unsurprising then that with the exception of some Hawaiian Drosophila species (Spieth
126 1981), it is difficult to observe any gross differences in segment position, size and
127 shape, scaled to full body size, between adults of different *Drosophila* species.
128 However, over longer evolutionary time scales, segment patterning can vary
129 substantially between arthropod species (Regier et al. 2010). At the early embryonic
130 stage in Drosophila, previous studies have shown small quantitative differences in
131 relative segment position between both closely related (Lott et al. 2007; 2010; Fowlkes
132 et al. 2011; Combs and Fraser 2018) and more distantly related species (Fowlkes et al.
133 2011; Wunderlich et al. 2018). Additionally, small quantitative differences were found
134 within a species when *D. melanogaster* lines were subjected to strong artificial selection
135 for egg size (Miles et al. 2010) and also between naturally occurring strains of *D.*
136 *melanogaster* (Jiang et al. 2015). It is not known, however, whether these changes
137 persist beyond the embryonic stage, or if these trends appear beyond the limited
138 number of species examined. So while segment patterning is well documented to be
139 robust, it has also been demonstrated to have some small quantitative level of variation
140 within and between species. Evolution of this robust trait does occur, and the presence
141 of some variation within species suggests that evolution of small changes may be
142 possible without causing catastrophic failure of patterning. Thus, we hypothesized that
143 the evolution of segmentation may occur by relatively small shifts, rather than rare large
144 leaps, which we would need large sample sizes to detect.

145 In order to investigate the evolution of segment patterning systematically across
146 a range of divergence times, we characterized segment position across 12 species of

147 *Drosophila*. The twelve species of Drosophila in our experiment spanned the
148 evolutionary history of the genus (40-60 million years; Russo et al. 1995; Obbard et al.
149 2012; Russo et al. 2013) and included three pairs of sister species. We measured
150 position of each abdominal segment relative to body length in the 1st instar larvae.
151 Larval stage is the latest developmental stage where all segments are visible and easy
152 to measure, and the use of this stage facilitated the measuring of hundreds of larvae
153 from each species. We found that relative segment positions at the larval stage have
154 changed many times in the evolutionary history of Drosophila, and that these changes
155 are mostly small in magnitude, with some larger changes. Most species-pair
156 comparisons showed differences in the relative position of most abdominal segments.
157 Some sister species were significantly different at every segment, however some of the
158 most diverged species in our dataset showed no differences in their patterning. The
159 magnitude of differences in relative position increased towards the posterior segments
160 in most species, most strikingly in *D. persimilis* and *D. mojavensis*. Phylogenetic
161 modelling showed that rate of segment position change was highly variable among the
162 branches along the Drosophila phylogeny. Results of a correlation analysis for position
163 change between segments within species suggested local control of segment position,
164 as correlations decrease with physical distance. Correlations for changes in the rate of
165 evolution of segment position between branches of the phylogenetic tree also
166 decreased with physical distance between segments. Overall, these results
167 demonstrate that this complex and robust developmental trait does evolve, even over
168 short timescales, and it does so primarily by small frequent steps with occasional large

169 leaps. This may permit the evolution of novel patterns over long periods of evolutionary
170 time, without compromising the integrity of this critical developmental phenotype.
171

172 ## Materials and Methods

173 *Species used*

174 We took relative position measurements of abdominal segments from 12 *Drosophila*
175 species spanning the evolutionary history of the genus. These species were *D.*
176 *melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. santomea*, *D. erecta*, *D.*
177 *ananassae*, *D. pseudoobscura*, *D. persimilis*, *D. willistoni*, *D. mojavensis wriglei* and *D.*
178 *virilis* (Figure 2A). We used the sequenced lines from 11 of these species (Clark et al.
179 2007). For *D. santomea* we used stock #14021-0271.01 (The National Drosophila
180 Species Stock Center).
181

182 *Fly husbandry and population control*

183 All flies were kept in plastic bottles in 20°C incubator with 60% humidity. Fly stocks were
184 raised on standard cornmeal food. The amount of time to sexual maturation varied
185 between species (Markow et al. 2008). Population control was conducted in a species-
186 specific manner through controlling the number of sexually mature adults added in a
187 given bottle, and how often the bottles were changed (Supplementary Table 1). As a
188 result, each bottle ended up with 40-50 pupae. Adult flies were discarded 14 days after
189 they became fertile, to control for parental age.
190

191 *Control experiment*

192 In order to address whether the larval mounting procedure has species-specific effects
193 on measurements of relative segment position in first instar *Drosophila* larvae, we
194 examined relative segment position in *D. melanogaster*, *D. sechellia* and *D. virilis* at
195 three different stages of the larval prep procedure (Supplementary Figure 1). First instar
196 larvae that hatched in distilled water were temporarily immobilized using a 3-5 minute
197 incubation on ice, put on a microscope slide with water and then imaged using a Zeiss
198 AxioImager microscope in brightfield. These larvae were then put back in distilled water
199 incubated at 60°C for 50 minutes and imaged in water a second time using a brightfield
200 microscope. The larvae were then mounted in PVA Mounting Medium (BioQuip) (see
201 next section). The larvae were then imaged a third time using dark field microscopy (see
202 "Imaging" section). In the images of the iced (untreated control, the icing procedure
203 slows larvae enough for imaging) and then the heated larva, each of the 2nd and 3rd
204 thoracic, plus eight abdominal segments were marked by a node between bulges on the
205 larval cuticle (Supplementary Figure 1). In the dark field images, segment borders were
206 determined by the anterior border of each denticle belt. Denticle belts are rows of
207 bristles on the ventral side of the larvae that are used for traction while crawling
208 (Bejsovec 2013). The anterior border of each denticle belt is a proxy for the anterior
209 border of each abdominal segment (Lohs-Schardin et al. 1979). Using the Image J
210 (version 1.47t) "Line tool", measurements were taken from the anterior end of the larva
211 to the border of each segment. Segment position was then determined by dividing this

212 value by the full length of the larvae. Segment position data was analyzed using the
213 following linear models, implemented in R (R Core Team, 2017).

214 $Y = \mu + prep + species + prep \times species + \varepsilon$.

215 $Y = \mu + prep + species + segment + prep \times species \times segment + \varepsilon$.

216

217 *Preparing Drosophila 1st instar larvae for imaging*

218 For each species, 20-50 newly emerged adults were obtained from a population-
219 controlled bottle and put in an egg collection bottle with a cap containing glucose-agar
220 food (100 grams of D-glucose, 15 gr of Agar, 1000 ml distilled H_2O with 1ml of Propionic
221 Acid added after autoclaving) that was sprinkled with Baker's yeast. For *D. sechellia,*
222 regular fly food was used in an egg lay cap with yeast sprinkled on it, as this prevents
223 this species from withholding egg laying, a particular problem with this species (Markow
224 et al. 2008). The bottle was then placed in 20°C incubator upside down. The next day
225 eggs were collected from egg lay caps, put onto a mesh and thoroughly washed using
226 distilled water to remove residual yeast and egg lay cap food. The eggs were then
227 placed in a petri dish filled with distilled water. This petri dish was placed in 20°C until
228 the eggs developed into first instar larvae. In addition to the variability in the number of
229 days necessary to reach sexual maturity, different species also varied in the number of
230 days necessary for a fertilized egg to develop into 1st instar larvae (Markow and
231 O'Grady 2005). The petri dish with water and larvae was then placed in 60°C oven for
232 ~50 minutes (Supplementary Table 1). This incubation killed and straightened the
233 larvae. These larvae were then mounted on standard glass slides using PVA Mounting

234 Medium (BioQuip), standard coverslips and a dissection microscope. Larvae were
235 oriented such that their ventral side was facing up, their posterior spiracles were
236 protruding from their body and their left-right symmetry was protected. Once the slides
237 were ready, each coverslip was sealed with clear nail polish. The slides were then
238 incubated at 60°C oven overnight.
239
240 *Imaging*
241 The slides were imaged at 40X objective using a Zeiss AxioImager microscope and a
242 dark field filter. Using automated tiling, 64 high resolution images were taken for each
243 larva, which were later stitched using the ZEN 2012 (blue edition) software. Each image
244 was then exported to "tagged image format".
245
246 *Image processing*
247 Measurements for the position of each abdominal denticle belt were made using a
248 custom Python script (the program will be made available prior to publication). This
249 program rotated and positioned each larva horizontally, anterior to the left and posterior
250 to the right, and cropped the image at the anterior, posterior and lateral borders. The
251 program then marked the anterior and posterior borders of the abdominal denticle belts.
252 Afterwards, it measured the distance from the anterior-most point of the larva to the
253 anterior as well as posterior borders of each denticle belt, and from the anterior-most
254 point of the larva to the posterior-most point of the larva. Relative segment positions
255 were calculated as the distance of the anterior denticle boundary from the anterior of the

256 larva, divided by larval length. The number of larval samples, from which segment
257 position measurements were taken and used for data analysis, varied from 105 to 145
258 for each species (Supplementary Table 1).
259
260 *Image editing*
261 Some of the images were edited using ImageJ "Brush" tool to paint over bubbles around
262 the larvae and to adjust brightness and contrast when necessary. We found both of
263 these edits increased the number of successful runs by the custom image processing
264 program and did not change the measurements taken from these images.
265
266 *Data analysis*
267 The segment measurement data is deposited in FigShare Repository at
268 https://doi.org/10.6084/m9.figshare.8170787. A linear model ANOVA was fitted to the
269 data using R (R Core Team, 2017) with the effects of species and segment and an
270 interaction term. The formula follows:
271 $Y = \mu + \text{species} + \text{segment} + \text{species x segment} + \varepsilon$
272 This was followed by Tukey's HSD (Honestly Significant Differences) (Steel et al. 1997)
273 test to conduct pairwise comparisons of the relative position of each segment between
274 species. Specifically, we used "HSD.test" function from the R package "agricolae". We
275 also replicated these results using t-tests between each species pair and then applying
276 Bonferroni multiple test correction for both multiple species and multiple segments. To
277 conduct these tests we used "t.test" and "p.adjust" functions in R.

For correlation analysis, we first calculated deviation from the between-species mean of the relative position of each segment in each species. We then used “cor” and “cor.test” functions in R to obtain Pearson correlation scores and the associated p-values.

Phylogenetic Ancestral State Estimation

We performed phylogenetic analyses to infer the evolution of relative segment position in the 12 *Drosophila* species in our study. For this, we first inferred an ultrametric phylogeny published in Turelli et al. (2018) under three candidate relaxed molecular-clock models. For each of the resulting phylogenies, we assessed the fit of four candidate models that variously describe how rates of segment evolution vary across branches of the phylogeny. Finally, we jointly inferred the phylogeny, model of segment evolution, and the ancestral states for each segment under the preferred model of segment evolution. We summarized various aspects of the evolution of relative segment position from the resulting joint posterior probability distribution of ancestral states. We provide complete details of these analyses in the Supplementary File 1.

Results

The larval preparation procedure does not have species-specific effects on segment position measurements

In order to test whether the larval preparation protocol had any species-specific effects on relative segment position measurements, we conducted a preliminary experiment using *D. melanogaster*, *D. sechellia* and *D. virilis*. These three species are

300 variable in their egg (Markow et al. 2008) and larval size (Figure 2C) and have varying
301 phylogenetic distances (Figure 2A). We therefore hypothesized that they might have a
302 higher chance of being differentially affected by the larval preparation protocol. We
303 imaged larvae from these species at three steps of the larval prep protocol: before
304 heating, after heating, and after mounting (See Methods). From these images we
305 manually took measurements of eight abdominal and two thoracic segments using
306 ImageJ (Supplementary Figure 1). Whether we included segment as a factor (p-value =
307 0.07 to 0.82) or not (p-value = 0.42 to 0.83) in our linear model formula, we did not find
308 any significant species-specific effects of the preparation protocol segment position
309 measurements.
310

311 **The dataset: larval segment allometry is highly variable across Drosophila**
312 **species**

313 To elucidate whether relative segment position has changed over *Drosophila*
314 evolutionary history, we compared relative abdominal segment position at the first instar
315 larval stage between 12 different species (Figure 2A). We found that there are many
316 significant differences in relative segment position among species (Figure 2B). Overall,
317 in 21 out of 66 species-pair comparisons, all eight segments had a significant difference
318 in their position. We note that species pairs do not represent independent observations
319 due to the underlying phylogeny (we perform a full phylogenetic analysis below). For the
320 majority of species-pair comparisons the relative position of five or more segments were
321 different (Supplementary Figure 2). In fact, there were only two pairs of species

322 compared (*D. simulans* - *D. ananassae* and *D. ananassae* - *D. willistoni*) where none of
323 the eight abdominal segments had a significant difference in their relative position
324 (Supplementary Table 2). Intriguingly, these species pairs are not closely related, with
325 15 and 32 million years of divergence, respectively. In all sister species comparisons in
326 our experiment, except for *D. yakuba* – *D. santomea*, the relative position of the majority
327 of abdominal segments were different. On the other hand, between divergent species
328 pairs, such as *D. melanogaster* and *D. virilis*, relative positions of only two segments
329 were different (Supplementary Table 2).

330 Next, we examined whether differences in segment position between species
331 were toward the head (anterior) or tail (posterior) of the larva. To do this, we calculated
332 mean positions of each segment across the 12 species in our study and characterized
333 whether a particular segment in a particular species were more anterior or more
334 posterior relative to this mean (Figure 3). We saw that, for the majority of the species,
335 the relative position of segments nearest each other differed in the same direction.
336 When the direction of the difference changed, it was between segments that had the
337 smallest (except for *D. willistoni*) magnitude of difference in a given species (Figure 3).
338 For seven out of 12 species, these differences were in one direction (anterior or
339 posterior) for all eight of their segments. In four species, *D. erecta*, *D. yakuba*, *D.*
340 *santomea*, *D. mojavensis,* the relative positions of all segments were more anterior,
341 whereas in *D. sechellia*, *D. ananassae* and *D. persimilis*, the relative positions of all
342 segments were more posterior as compared to the mean position for each segment
343 over 12 species. In *D. simulans* and *D. pseudoobscura*, there was only one segment

344 that was more anteriorly positioned, the rest of the segments were more posteriorly
345 positioned as compared to the mean position across the 12 *Drosophila* species in our
346 study.

347 Notably, averaged over all species, deviation from species mean in relative
348 segment position appeared to steadily increase toward the posterior of the larvae
349 (Supplementary Figure 3A). This trend was largely, but not exclusively, driven by the
350 segment position shifts in *D. mojavensis* and *D. persimilis* (Figure 3, Supplementary
351 Figure 3A, B), which had the largest difference for segment A8 (which extends from the
352 anterior boundary of A8 to the tail of the larvae; we will refer to this segment as A8+tail)
353 among all the species. *D. mojavensis* has the largest magnitude of difference from the
354 mean over all species, with a much longer A8+tail segment, while *D. persimilis* has an
355 exceptionally short A8+tail segment. (Figures 1 and 2, Supplementary Figure 5C). We
356 also compared the rate of increase in magnitude of difference in relative segment
357 position along the length of the larvae between species. We saw that the steepest
358 increase was in *D. mojavensis*, followed by *D. persimilis*, *D. erecta* and *D.*
359 *pseudoobscura* (Figure 3, Supplementary Figure 3B). Interestingly, in all of the species
360 except for *D. sechellia*, *D. yakuba* and *D. willistoni*, magnitude of difference was highest
361 for the relative position of segment A8+tail. On the other hand, total number of
362 significant differences in relative position observed in species-pair comparisons did not
363 change much for segments A3 through A8, with segments A1 and A2 showing a slightly
364 lower number of significant differences in relative position (Supplementary Figure 4B,
365 black line).

366

367 **Changes in the length of larval body, head, thorax and tail**

368 We compared whole body length at the first instar larval stage among 12
369 *Drosophila* species (Figure 2C). *D. santomea* was the largest at this stage, followed by
370 *D. sechellia* and *D. virilis*. *D. perimilis* and *D. pseudoobscura* were the smallest,
371 followed by *D. mojavensis*. Body length for the rest of the species showed few and
372 smaller differences (Figure 2C). We had two comparisons between sister species with
373 large size differences, as *D. santomea* and *D. sechellia* were among the largest larvae,
374 while their sisters, *D. yakuba* and *D. simulans*, respectively, were of roughly average
375 size. Sister species *D. santomea* and *D. yakuba* had few differences in segment
376 position, whereas *D. sechellia* and *D. simulans* were different for every segment,
377 suggesting that larval size is not predictive of segment position differences. Overall,
378 there was no correlation between change in body length and the number of differences
379 in relative segment position between species (Supplementary Figure 5A). In other
380 words, having a bigger difference in body length was not correlated with having more
381 differences in relative segment position between species. Moreover, we detected no
382 difference between distributions of body lengths according to the number of more
383 anterior versus more posterior segments (Wilcoxon test, p-value=1). Thus, having a
384 shorter or a longer body was not correlated to having a bias towards higher number of
385 more anterior or more posterior segments.

386 We also compared the relative size of the head and thorax region (we will refer to
387 this region as h+t), i.e., head and three thoracic segments, normalized to the whole

388 body, among 12 Drosophila species and found only a few differences (Supplementary
389 Figure 5B). *D. virilis*, which is the third largest species at the first instar larval stage, had
390 the smallest h+t segments. The biggest h+t segments belonged to *D. persimilis*, the
391 smallest species and to *D. sechellia*, the second largest species at the first instar larval
392 stage. For the rest of the species, relative size of h+t seemed to fall in between, with
393 only few significant differences. Contrarily, we found many significant differences in the
394 relative size of A8+tail segment (Supplementary Figure 5C). Besides the largest and
395 smallest A8+tail segments observed in *D. mojavensis* and *D. persimilis*, respectively,
396 there are many differences of smaller magnitude between the rest of the species. We
397 also examined the width of our segment marker, the rows of bristles known as denticle
398 belts (see Methods) and re-performed our analysis for the posterior of the denticle belt
399 rather than the anterior. Briefly, the denticle belt marking segment A1 was the narrowest
400 (and most variable), with A8 being second narrowest, with the rest being of similar size,
401 with these patterns holding across species (Supplementary Figure 8A). Measuring
402 segment position from the posterior border of the denticle belt, we obtained very similar
403 results to those reported for the anterior border (Supplementary Figure 8B, C). For
404 details of these analyses see Supplementary File 2.
405

406 **Changes in the size of A8+tail segment are responsible for a significant portion of**
407 **the shifts in relative segment position between species**

408 Given that the relative position of segment A8+tail, as compared to the other
409 segments, had the largest deviation from the species experiment-average for most of

410 the species in our study (Supplementary Figures 3A, Supplementary Table 3) we asked
411 how much of the total variation in relative segment position is driven by A8+tail. In order
412 to address this, we recalculated relative segment position in the absence of A8+tail (see
413 Supplementary File 3). To determine that any changes in segment position we see was
414 due to the removal of A8+tail region specifically and not simply due to the removal of an
415 end segment, we made a separate recalculation of relative segment position after
416 removing the h+t region. In both cases we determined the total number of significant
417 differences in relative segment position between pairs of species and corrected it
418 according to the number of segments that were used in position calculations. We found
419 that the corrected total number of significant position differences was reduced from
420 51.25 to 38.42 (~25% decrease) when A8+tail was removed and increased from 51.25
421 to 53.71 when h+t was removed (Supplementary Figure 4A, compare also Figure 2B to
422 Supplementary Figure 6A and B). This suggests that changes in the size of A8+tail
423 drove a substantial portion of the differences in relative segment position between
424 species. On the other hand, changes in the size of h+t appear to have masked some of
425 the inter-species differences in relative position observed in the rest of the segments.
426 These results are consistent with the finding that the average difference in relative
427 position of A1 are the smallest in magnitude among all segments (Supplementary
428 Figure 3A), while A8 has the largest magnitude changes. For a more detailed analysis
429 of "end removal" and its effects see Supplementary File 3.
430
431 **Is the A8+tail segment more variable within lines than the other segments?**

432 As the posterior terminal segment (A8+tail) has the largest differences in
433 magnitude in our dataset and may be driving a significant proportion of the differences
434 we observe, we asked whether this segment was also the most variable within lines. To
435 determine if segment positioning in the anterior of the embryo is less noisy as compared
436 to the posterior, we measured coefficient of variation for the position of each segment
437 across species. When all segments are present in position calculations, then the
438 coefficient of variation averaged across all species does not vary considerably across
439 the length of the larva and stays low (~0.03) (Supplementary Figure 7A). This suggests
440 that while this segment has the largest magnitude differences in our dataset, it is not
441 positioned any less precisely.
442
443 **Phylogenetic analysis shows variable rates of evolution**
444 To investigate how segmentation has evolved over *Drosophila* evolutionary
445 history, we implemented phylogenetic methods to estimate the rates of morphological
446 evolution over the phylogeny. We employed a multivariate Brownian motion model of
447 evolution (Huelsenbeck and Rannala 2003; Lartillot and Poujol 2010), and tested
448 various morphological branch rate prior models that describe how rates of
449 morphological evolution vary across the branches of the tree. These analyses were
450 performed in RevBayes (v. 1.0.7; Höhna et al. 2016), and are outlined in detail in
451 Supplementary File 1. Of the candidate models of segment evolution that we explored,
452 the data significantly preferred the uncorrelated lognormal relaxed molecular clock
453 model, which allowed rates to vary episodically along ancestor-descendant branches

454 (*i.e.*, rates of segment evolution are not correlated between ancestor-descendant
455 branches). All the results in the main text are for this model, but other morphological
456 evolutionary models gave similar results (Supplementary File 1).

457 Our phylogenetic analyses indicate that evolutionary rates of relative segment
458 position are highly variable across branches of the *Drosophila* phylogeny (Figure 4A,
459 Supplementary Figure 9A, B). Many of the highest rates of evolution are on branches
460 for species with sister species included in the analysis (i.e. *D. sechellia* and *D. simulans*;
461 *D. santomea* and *D. yakuba*; *D. persimilis* and *D. pseudoobscura*). This points to
462 segment position evolving quickly between these sibling species. The patterns vary
463 among segments (Supplementary Figure 10). The rate of *D. sechellia* evolution
464 compared to *D. simulans* (and the rest of the species) is highest for the first four
465 abdominal segments (Supplementary Figure 10). The rate of evolution for *D. santomea*
466 is highest relative to *D. yakuba* in the center of the larvae, segments 4 and 5
467 (Supplementary Figure 10). Two lineages that are also evolving rapidly (Figure 4A),
468 those leading to *D. mojavensis* and *D. persimilis*, have the longest and shortest
469 posterior-most segment (A8+tail), respectively, in our dataset (Figure 2B,
470 Supplementary Figure 5C). The inclusion of the long branch leading to *D. mojavensis*
471 among branches with the fastest rates of evolution is of interest, because the other
472 lineages that we found to be evolving quickly are on shorter branches. This high rate
473 sustained over a long branch has resulted in the A8+tail segment of *D. mojavensis*
474 having the largest magnitude change in our dataset (Figure 2B, Supplementary Figure
475 3B). The rate of segment evolution in *D. mojavensis* and *D. persimilis* is highest in the

476 posterior half of the larvae (Supplementary Figure 10), giving rise to the large
477 magnitude of changes in the A8+tail segment (Figure 2B, Supplementary Figure 3B).

478 Given that several species had large changes in segment position toward the
479 posterior of the larvae (Figure 2B, Figure 3, Supplementary Figure 3B), we asked
480 whether the overall rate of evolution is also higher for some segments than it is for
481 others. We examined the rate of position evolution for each segment over all branches
482 of the tree. The evolutionary rate of relative segment position varies among segments,
483 with posterior segments exhibiting elevated rates (Figure 4B). Segment A8 had the
484 highest rate of change in segment position, with segment A7 also having a slightly
485 elevated rate (Figure 4B). The elevated rate of evolution for A8 is consistent with some
486 of the largest magnitude changes in our raw data (Figures 2B, 3) being found for this
487 segment.

488 To examine the magnitude of shifts in segment position, we examined the
489 distribution of the amount of change in relative segment position normalized across all
490 branches of the tree (i.e. amount of change per unit time; see Supplementary File 1,
491 S.3.4.5 and S.3.4.6 for details). The distribution of normalized magnitude of change in
492 segment position across all the branches of the phylogeny (Figure 4C) shows that most
493 segment positions on most branches have experienced small magnitude changes,
494 whereas some segment positions on some branches have experienced substantially
495 larger changes within a given amount of evolutionary time.

496

497 **Is segment position along the anterior posterior axis controlled locally or**
498 **globally?**

499 Within each species, we asked how the shift in the relative position of one
500 segment might be correlated to shifts in the relative position of other segments. This
501 would show whether there is a focal point along the anterior-posterior axis controlling
502 segment position or whether there is a more complex underlying regulatory mechanism.
503 We found that within all of our species, the correlation between shifts in the relative
504 positions of any two segments is inversely proportional to the distance between the two
505 segments (Figure 5A, Supplementary Figures 11, 12). We did not observe a focal point
506 governing the shift in segments, where it might be expected that correlations would drop
507 off as the distance from that particular point increased. It appears that small
508 adjustments in segment position are made locally. These correlations are looking at the
509 relationship between segment positions across individuals within a species. As the
510 variation within species reflects developmental variation in a genetically identical line,
511 these correlations show that developmental variation in the positioning of one segment
512 have stronger effects on nearby segments than more distant segments. This is
513 consistent with evidence from the embryonic stage that showed correlation between
514 deviation from mean in stripe boundary for various segmentation genes went down with
515 increasing distance between their patterning domains of segmentation genes (Lott et al.
516 2007). Interestingly, averaged over all species, shift in the relative position of A8 was
517 less well-correlated to shifts in the rest of the segments (Figure 5B). This relationship
518 was strongest when adjacent segments were compared (Figure 5B), and less strong as

519 the distance between segments being compared increased (Supplementary Figure 13).
520 This suggests that shifts in the relative position of A8 are more independent than the
521 shifts in the relative position of other segments within species. For a report of correlation
522 scores between adjacent segments along the anterior-posterior axis for each species
523 see Supplementary File 4.

524 Next, we asked, given that the relative position of A8+tail changed more
525 independently within a species as compared to the other segments (Figure 5B), would
526 segment position be more tightly regulated in the absence of A8+tail or is A8+tail
527 essential for proper segment positioning? To address this question, we calculated
528 correlation scores using relative segment positions determined in the absence of
529 A8+tail. We saw that the absence of A8+tail resulted in lower correlation scores
530 between position shifts of adjacent segments as compared to when all segments were
531 present in position calculations. (Supplementary Figures 16 and 18, red line, *D.*
532 *sechellia* was exceptional, see Supplementary Figure 17). Similarly, when we removed
533 h+t from position calculations, averaged over all species, the correlation between
534 position shifts of adjacent segments was reduced as compared to when all segments
535 were present in position calculations. (Supplementary Figures 16 and 18, blue line).
536 These results suggest that both ends of the larvae are needed for proper segment
537 positioning, and removal of either one decreases the level of coordination between
538 segments.

539

540 **Coordination of segment position evolution across the larva**

541 Within a species, segment position seems to be controlled locally in response to
542 developmental variation, as correlations between segments decrease with physical
543 distance in the larva. How then are evolutionary segment position changes coordinated
544 across species? To address this question, we used the phylogenetic model to correlate
545 the rate of segment position change across species, across the larva. These
546 phylogenetic correlations may reflect underlying genetic correlations (in a quantitative
547 genetic sense) or be the result of constraints placed on the system by selection
548 pressures. Evolutionary rates of relative segment position are significantly correlated
549 across the branches of the phylogeny, and correlation decreases as the anatomical
550 distance between segments increases (Figure 5C). Additionally, all correlations are
551 positive, indicating that, for example, when rapid evolution occurs in one segment,
552 changes occur across all segments. However, across species, the rate of evolution of
553 segment A8 is not less correlated than expected, given its distance from other
554 segments. This is interesting to consider in light of the observation that within species
555 A8 is less correlated to the other segments than expected given its anatomical distance,
556 and perhaps surprising given that A8 also possesses a higher rate of evolution than the
557 other segments (Figure 4B). While this segment may evolve at a faster rate, on average
558 it does not seem to be evolving in a less coordinated fashion than the other segments,
559 when the whole phylogeny is considered.
560

561 **Discussion**

562 Many critical developmental processes and traits are known to be robust, in that
563 they are produced faithfully despite variation encountered in ontogeny. This tolerance of
564 variation does have limits, however, and exceeding these limits may disrupt the
565 development of these traits in such a way that development does not proceed. Hence,
566 robustness is desirable during development because it assures the precise production
567 of critical traits and processes within a range of developmental conditions.

568 Given the suppression of phenotypic variation in robust traits, their evolution has
569 been a subject of considerable interest to researchers over the years, and has produced
570 both theoretical and empirical work (Wagner 2005; Félix and Wagner 2006; Masel and
571 Siegal 2009; Siegal and Leu 2014; Payne and Wagner 2019). One proposal for the way
572 for a robust trait to evolve is for conditions to exceed the tolerance of variation for that
573 trait, and expose genetic variation that had previously been masked, i.e. cryptic genetic
574 variation, by the very robustness of the trait (Rutherford and Lindquist 1998; Gibson and
575 Dworkin 2004; Paaby and Rockman 2014). Unraveling any amount of genetic variation
576 of unknown consequence to a critical trait at a time of great stress seems like a
577 dangerous proposition in a multicellular animal.

578 Here, we focus on a well-studied robust process and trait, segmentation in
579 *Drosophila*. Previous studies have highlighted the ability of the segmentation network to
580 produce precisely localized segment markers in embryogenesis despite experimentally
581 produced perturbations in development (Félix and Wagner 2006; Masel and Siegal
582 2009). Our study extends this to measure the trait of segmentation in larvae, across 12
583 Drosophila species. Here, we demonstrate that segmentation varies considerably

584 across species, and that the rate of evolution of segment position varies considerably as
585 well. The variation in segment position consists mostly of small-magnitude differences,
586 suggesting that this trait evolves by many small steps, with some occasional larger-
587 scale changes. While it is difficult to compare sections in the embryo to segments in the
588 larva, this result is consistent with patterning differences observed between species in
589 the embryo (Lott et al. 2007; 2010). Our data shows that segment position differences
590 exist between species in later developmental stages as well. Additionally, we find that
591 each line of each species measured here precisely produces its characteristic
592 segmentation pattern in the larva. This points to the ability of segmentation to evolve
593 without losing its ability to be precisely localized (Lott et al. 2010). While we did not test
594 the robustness of each species to genetic and environmental variation specifically, this
595 suggests that segmentation can evolve and remain robust to stochastic variation. If
596 segmentation can evolve small quantitative changes over evolutionary time without
597 losing robustness, then this suggests that not all genetic variation in this trait is cryptic
598 (Miles et al. 2010; Jiang et al. 2015), and small-scale differences (and the occasional
599 larger-scale difference) in segmentation patterns may be available to selection (Weber
600 1992). Or, perhaps there is a neutral space where a range of segment positions are
601 tolerated, and stabilizing selection keeps them in that range.

602 The evolutionary patterns observed here may also be simply what might be
603 expected of complex traits generally, with changes in a number of genes producing
604 small quantitative changes in phenotype over evolutionary time. Here, we find that
605 segment position seems locally determined along the length of an individual, as position

606 shifts are highly correlated between local segments, and correlations drop off over
607 distance, consistent with a previous result within species in embryos (Lott et al. 2007).
608 This is also consistent with the known regulatory interactions in this network, where
609 patterns are refined over developmental time (Manu et al. 2009a) and regulation of
610 segment patterning becomes more local at each stage as compared to the previous one
611 (Nüsslein-Volhard and Wieschaus 1980; Kornberg and Tabata 1993; Nüsslein-Volhard
612 et al. 2008). This result suggests that a number of genes are involved in producing the
613 differences observed between species. This makes it difficult to determine the identity of
614 individual genetic changes that may cause the differences in segment position.

615 Across the larva, the most striking pattern we observed can be found in the
616 posterior-most segment, from A8 to the tail of the animal. We find that this segment has
617 the largest magnitude of differences between species. It has a higher rate of evolution
618 between species than the other segments. It is the most independently controlled
619 segment within a species, with correlations between its position and all other segments
620 being the lowest found. As the h+t region anterior of A1 does not share these properties
621 in our study, it is not simply a feature of terminal segments, but specific to the A8+tail
622 region. The A8+tail region contains the posterior spiracles, important breathing
623 structures in the larva (Hu and Castelli-Gair 1999), as well as the genital imaginal disc
624 from which the genital structures in the adult are produced (Sánchez and Guerrero
625 2001). While genital structures are known to evolve rapidly between species (Eberhard
626 2013), it is unclear whether this would produce differences in the entire segment in
627 which the genital imaginal disc is found. Alternatively, the differing conditions in which

628 larvae find themselves may produce differences in behavior that would require
629 differences in posterior spiracle length. We are currently exploring this possibility in
630 relatives of *D. mojavensis*, as this species has an exceptionally long A8+tail segment. In
631 our care, *D. mojavensis* larvae burrow deeply in food with only their long posterior
632 spiracles above the surface of the food. As *D. mojavensis* and its relatives are desert-
633 dwelling cactus specialists (Oliveira et al. 2012), perhaps the larvae burrow deeply into
634 cactus in their natural environment to find a more hospitable microclimate (McKenzie
635 and McKechnie 1979; Green et al. 1983).

636 Overall, our results show for the first time that segment position in Drosophila has
637 changed frequently throughout the evolutionary history of the genus. The changes were
638 mostly small in magnitude, presumably representing only small perturbations to the
639 development of the organism. Rate of evolution of this trait varies across the phylogeny,
640 with larger magnitude differences and a higher rate of evolution observed for the tail of
641 the larva. Changes in segment position within species, as well as rates of evolution
642 between species, are highly coordinated with changes in the closest segments,
643 indicating the highly coordinated nature of the genetic network underlying this trait.
644 Future studies are needed to unravel the nature of the genetic changes underlying
645 segment position differences between species and whether any of the observed
646 phenotypic differences between species might be adaptive.

647

648 **References**

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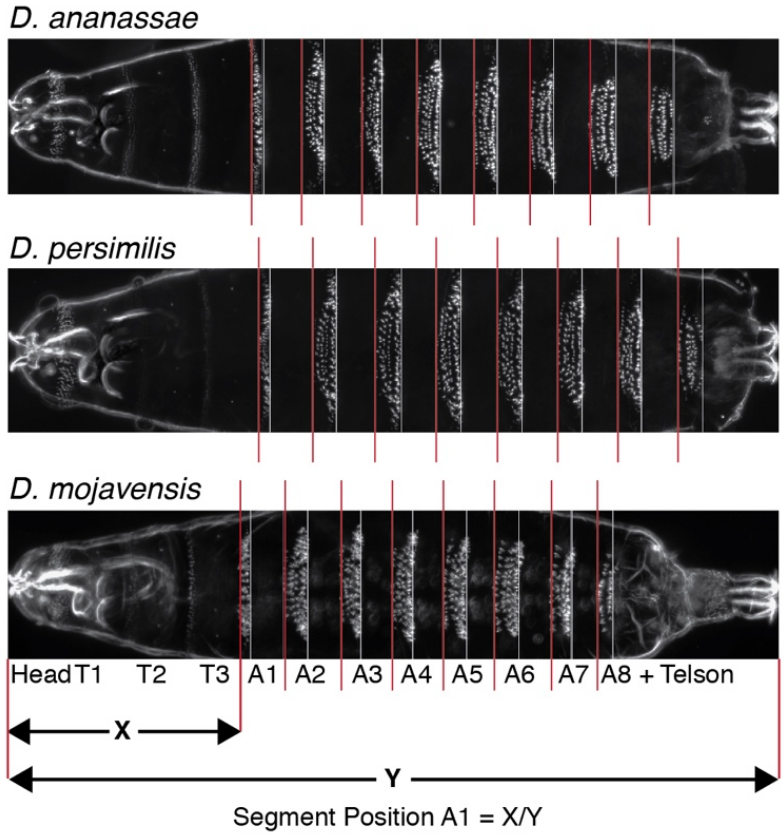
Figure 1



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869
870 **Figure 1.** Dark field images of first instar larvae from three species mounted as
871 described in Materials and Methods. Red lines mark the anterior border and white lines
872 mark the posterior border of abdominal denticle belts. Denticle belts were recognized by
873 our image processing program. The anterior border was used as a proxy for segment
874 border. To measure the position of an abdominal segment (e.g. A1) relative to the body
875 length (Y), the distance from the anterior border of the larvae to the anterior border of
876 that segment (X) is divided by the total body size (Y). T = Thoracic Segment, A =
877 Abdominal Segment.
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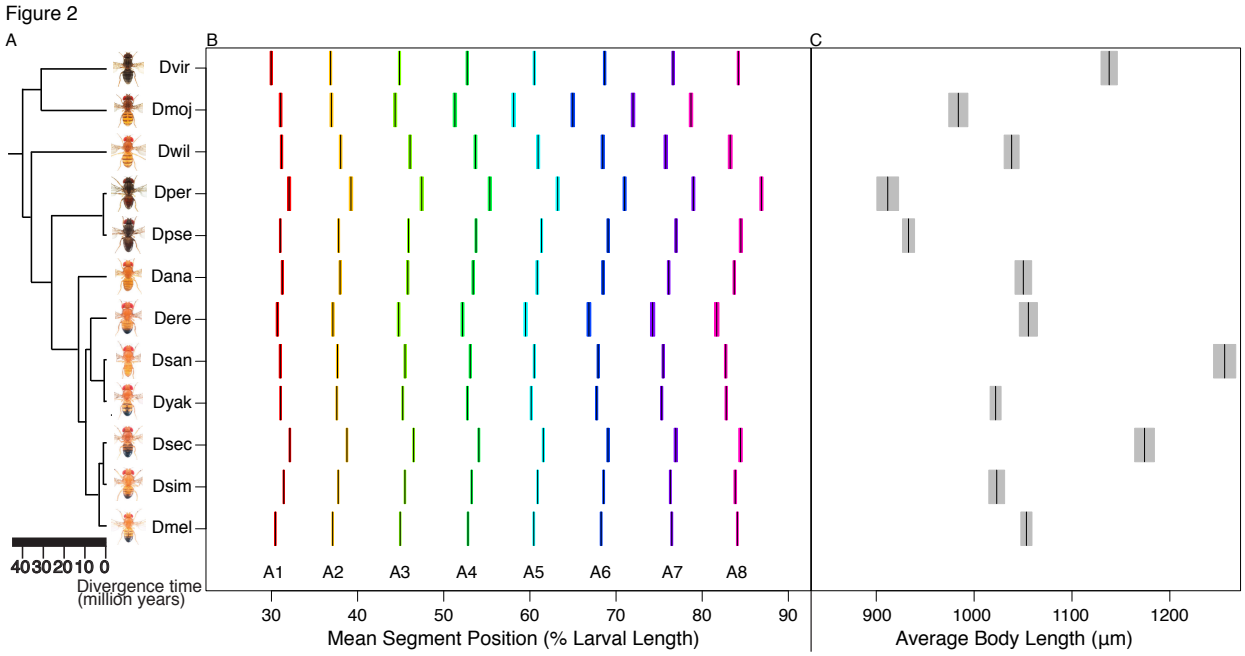


Figure 2. Twelve Drosophila species show many differences in relative segment position and total body length. (A) Twelve Drosophila species on an evolutionary tree. Adult male species pictures were kindly provided by Nicolas Gompel and were downloaded from FlyBase. (B) Each black bar represents the mean position in percent larval length for each segment in each species. The color spread on the two sides side of each black bar is 95% confidence interval. Each segment is represented by a different color. x-axis is relative segment position in percent larval length. A = Abdominal Segment. (C) Each black bar represents the average first instar larval body length for each species. The gray shaded area around the black bars is 95% confidence interval. x-axis is average body length in micrometers.

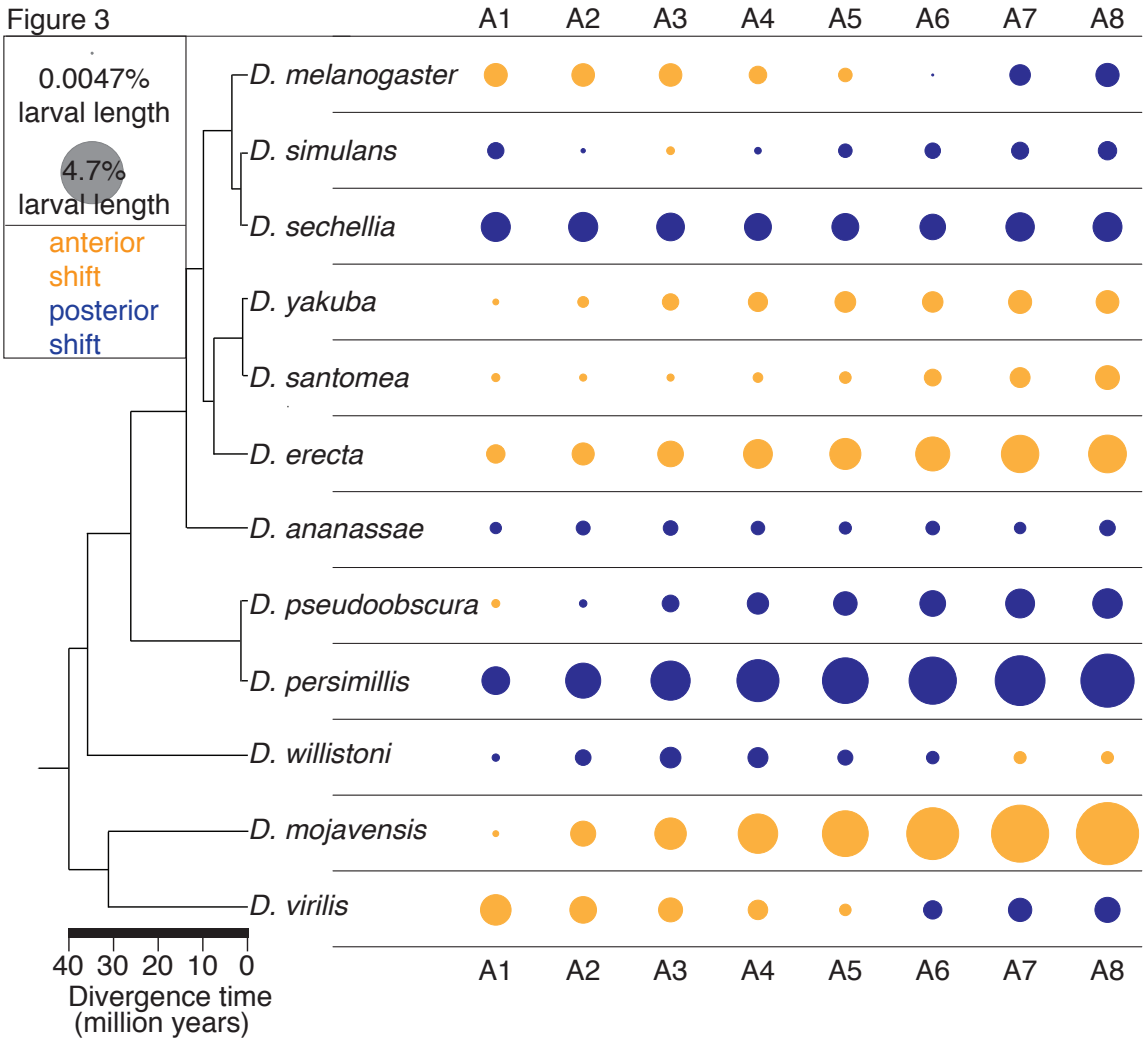


Figure 3. Blocks of segments tend to shift together in a certain direction rather than alternating segments shifting in opposite direction. Yellow indicates anterior, blue indicates posterior shift in relative segment position as compared to the species mean for a given segment position. The area of the circle is proportional to the size of the shift in relative segment position as compared to the species mean for a given segment position.

Figure 4

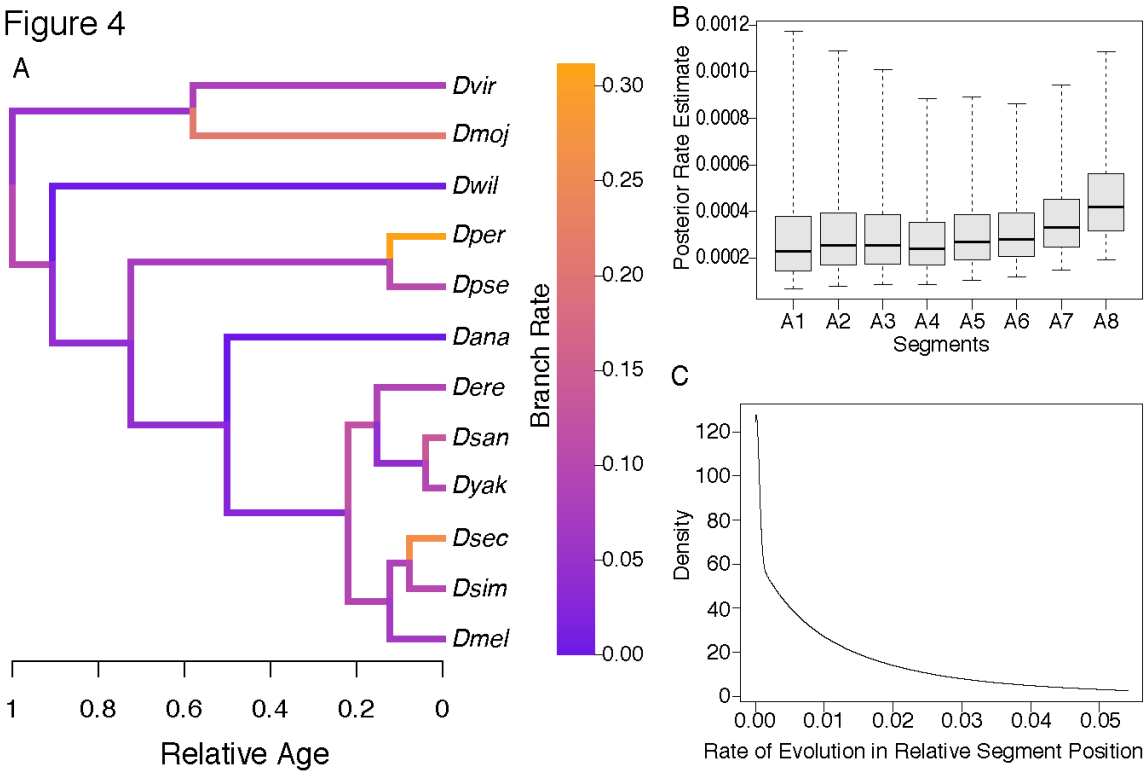


Figure 4. Phylogenetic analysis of segment evolution in the 12 *Drosophila* species. (A) Phylogeny inferred from nuclear loci with relative divergence times. Branches of the tree are colored to indicate the overall rate of relative segment position evolution. (B) Boxplots indicate the posterior distribution of the evolutionary rate of relative segment position. The bar indicates the posterior mean rate; the boxes and whiskers indicate the 50% and 95% posterior credible intervals, respectively. (C) Marginal posterior probability distribution of the evolutionary rates of relative segment position across branches.

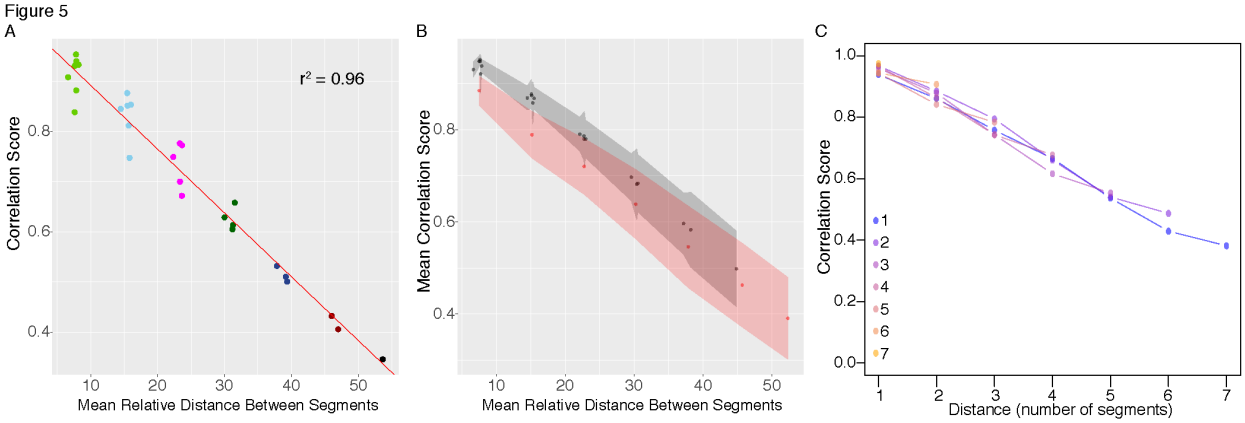
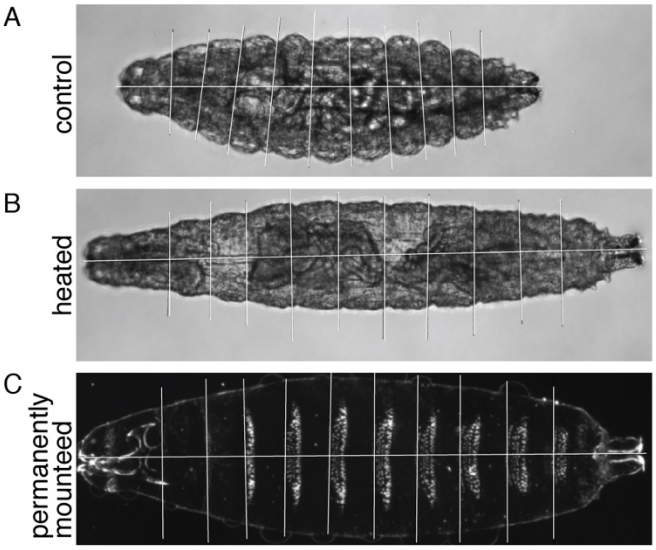


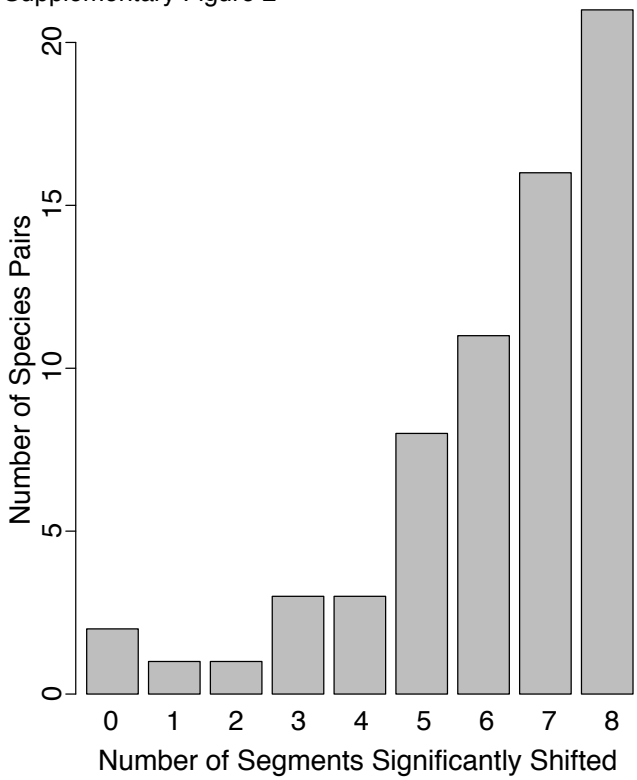
Figure 5. Correlations between segments, within and between species. (A) Within a species, correlation between the shift in the relative position of any two segments goes down as the distance between them gets bigger. The sample graph is for *D. melanogaster*. Colors are used to distinguish between segment pairs with different number of segments in between. (B) Over all species, average correlation score between the shift in the relative position of any two segments goes down as the distance between the two segments get bigger. Among all abdominal segments, A8 tends to have a worse correlation score with any given segment regardless of its distance to the other segment. Black dots represent the segment pairs that do not contain A8, red dots represent the segment pairs that contain A8. The gray and red shaded areas around the black and red dots, respectively, is their 95% confidence interval. The unit of the x-axes is in panels A and B is percent larval length. (C) Between species, correlations between evolutionary rates for a pair of segments are lower with increasing distance between segment pairs. Color indicates the first segment in the comparison, with the most blue indicating comparisons with segment 1, and the most yellow being comparisons with segment 7.

Suplementary Figure 1



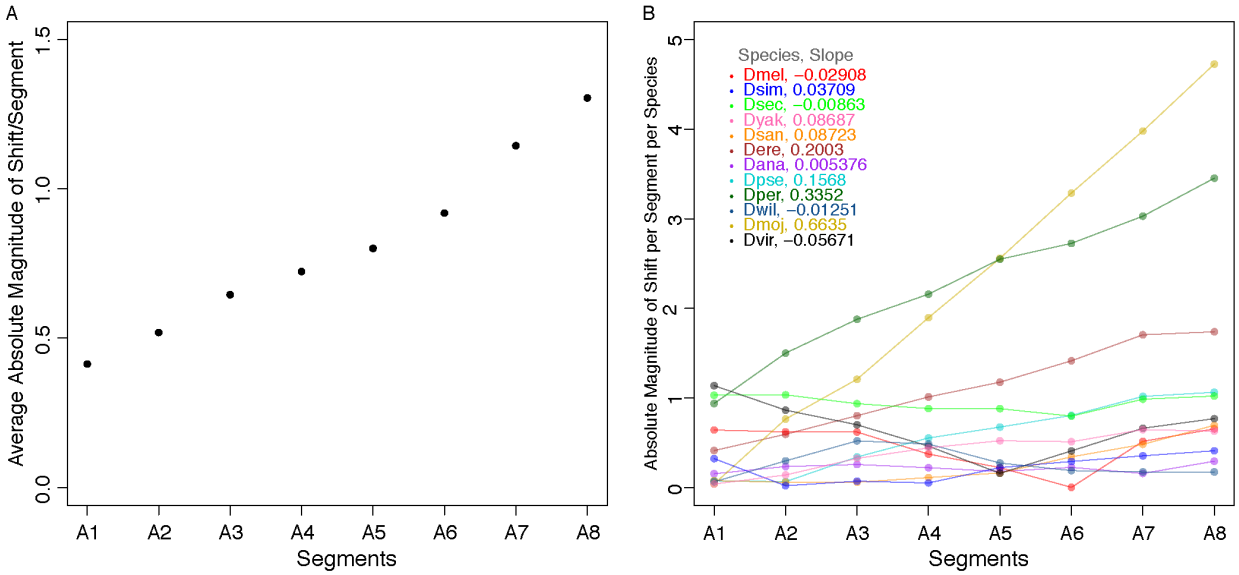
Supplementary Figure 1. (A) Bright field image of a *D. melanogaster* first instar larva, hatched in water, iced for 2-3 minutes before taking a picture. (B) The same *D. melanogaster* larva heated at 60°C for an hour prior to taking a bright field picture. (C) Dark field image of the same *D. melanogaster* larva, mounted using PVA, and incubated in the 60°C oven overnight. White horizontal lines mark the full length of the larvae, vertical lines mark the segment borders.

Supplementary Figure 2



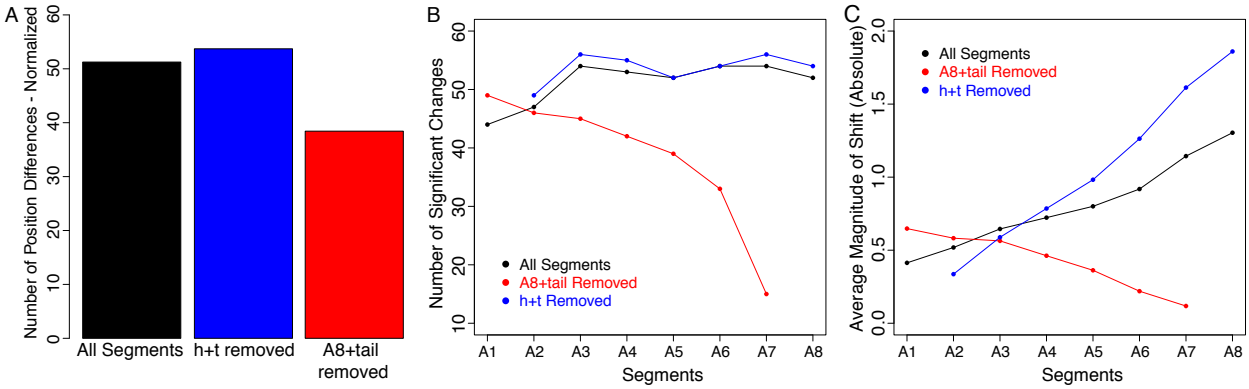
Supplementary Figure 2. For the majority of species pair comparisons, five or more segments had significantly shifted relative positions. The graph shows the number of species pairs on the y-axis and number of segments significantly shifted on the x-axis.

Supplementary Figure 3

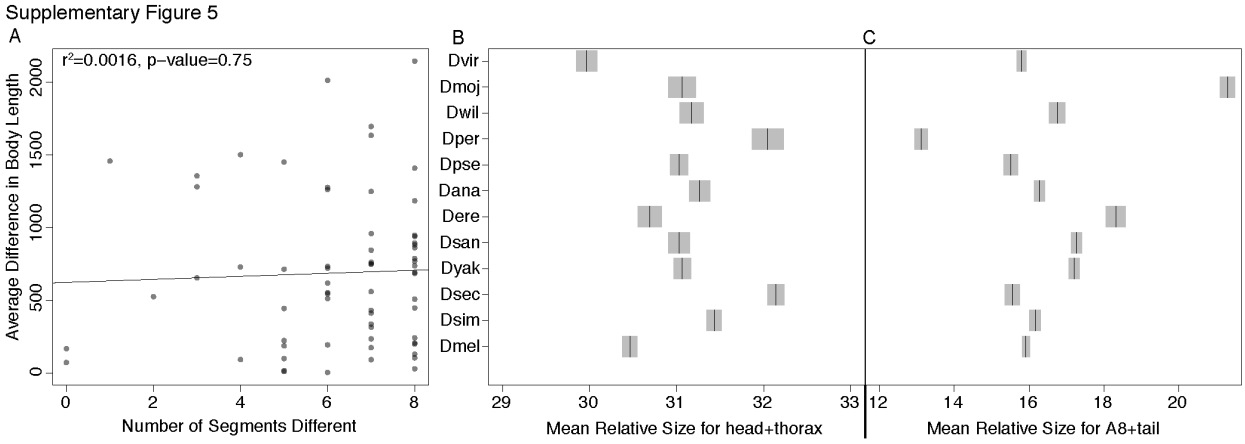


Supplementary Figure 3. (A) Absolute magnitude of shift in relative segment position as compared to the species mean for each segment position increases from anterior to the posterior of the larvae. On the y axis of this graph is absolute magnitude of shift, in percent larval length, for each segment averaged over all species, on the x axis are abdominal segments A1 through A8. (B) Absolute magnitude of shift changes from anterior to posterior differently for each of the 12 Drosophila species. The graph shows absolute magnitude of shift in percent larval length on the y axis and abdominal segments A1 through A8 on the x axis. The legend shows the slopes calculated for each trend form anterior to posterior. Lines in the graph and the legend are color coordinated for each species.

Supplementary Figure 4

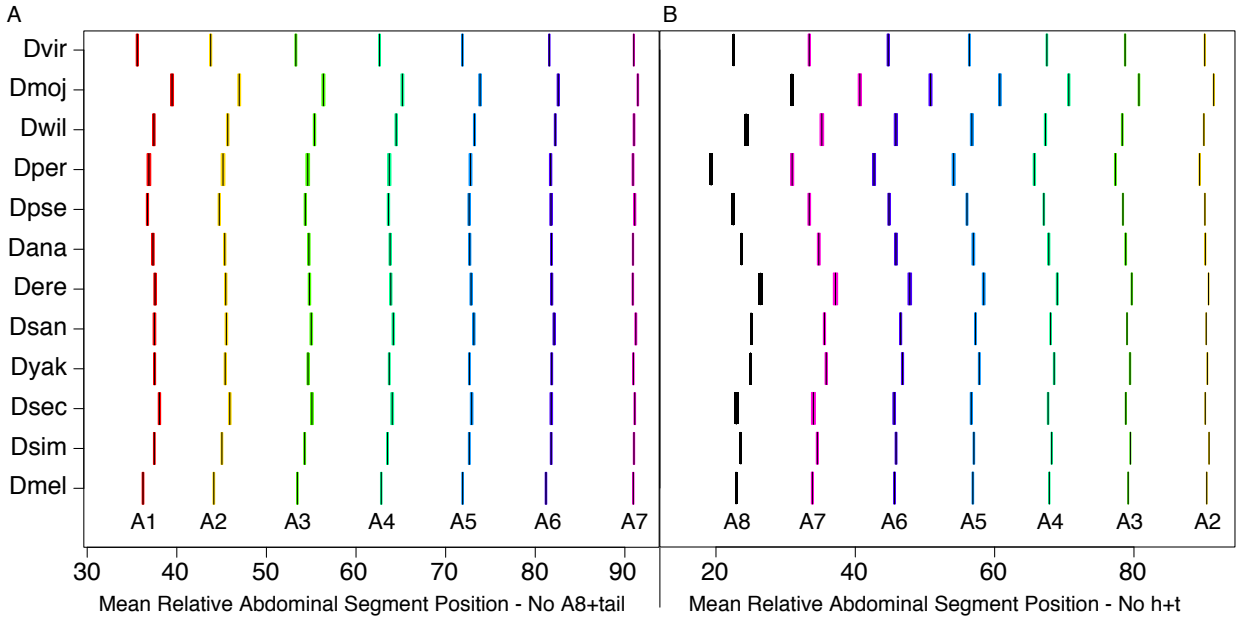


Supplementary Figure 4. (A) Comparison of significant position changes when segment positions were calculated with all segments included, with h+t removed or with A8+tail removed. Removal of A8+tail shows a decrease, whereas removal of h+t shows an increase, in the number of segments that have significantly changed their relative position as compared to when all eight abdominal segments were included in the position calculations. y-axis is total number of significant changes, normalized to the number of segments included in position calculations. (B) When A8+tail was removed from position calculations, number of significant changes in relative segment position went down sharply towards the posterior end of the larvae. However, when h+t was removed in the same manner, number of significant changes in relative segment position followed the same pattern from anterior to posterior as when all abdominal segments were included in position calculations. y-axis is total number of significant changes in segment position. (C) Average magnitude of shift (from the species mean) for each segment went down sharply towards the posterior of the larvae when A8+tail was removed from segment position calculations. On the other hand, removing h+t followed the same pattern as when all segments were included in calculations, but showed a bigger deviation from the species mean towards the posterior of the larvae. y-axis is the average magnitude of shift (from the species mean) in segment position, in percent larval length, ignoring the direction. x-axis is abdominal segments in both panels B and C.



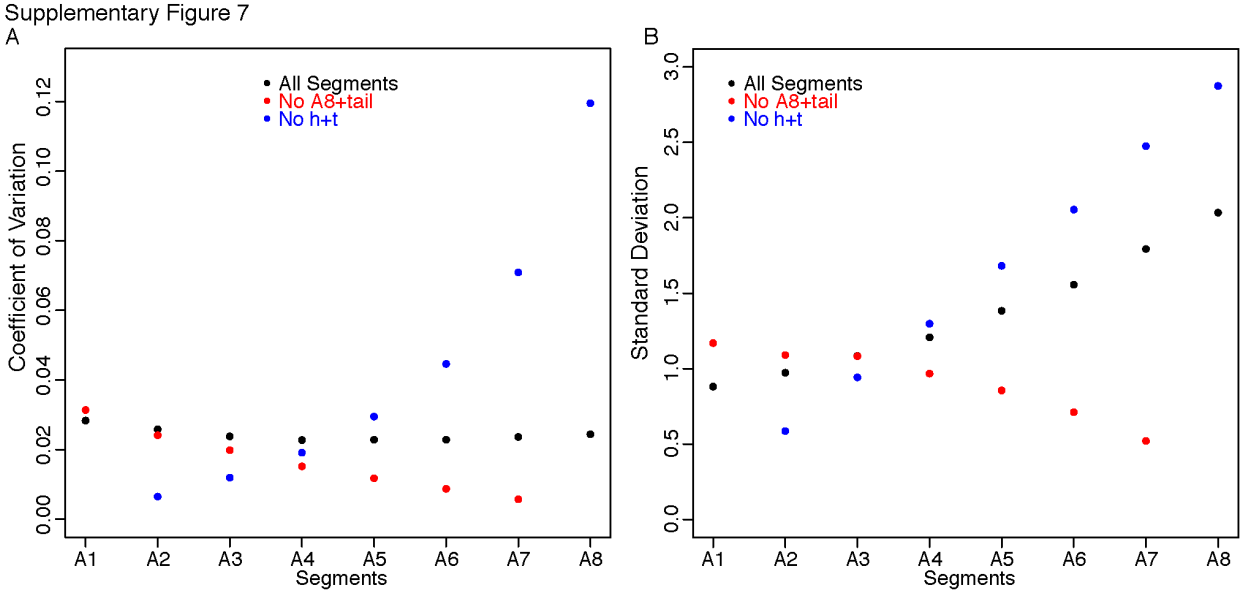
Supplementary Figure 5. (A) Average body length difference between a given pair of species is not proportional to the number of segments with significant position shifts. y axis in this graph shows average difference in body length (in pixels) between each pair of species and the x axis shows the number of segments with significant position shifts in the same species pair. (B) This graph shows differences in mean relative size for head+thorax, in percent larval length, among 12 Drosophila species. The black bars indicate the mean and the gray shaded area is 95% confidence interval for each mean. (C) This graph shows differences in mean relative size for A8+tail, in percent larval length, among 12 Drosophila species. The black bars indicate the mean and the gray shaded area is 95% confidence interval for each mean.

Supplementary Figure 6

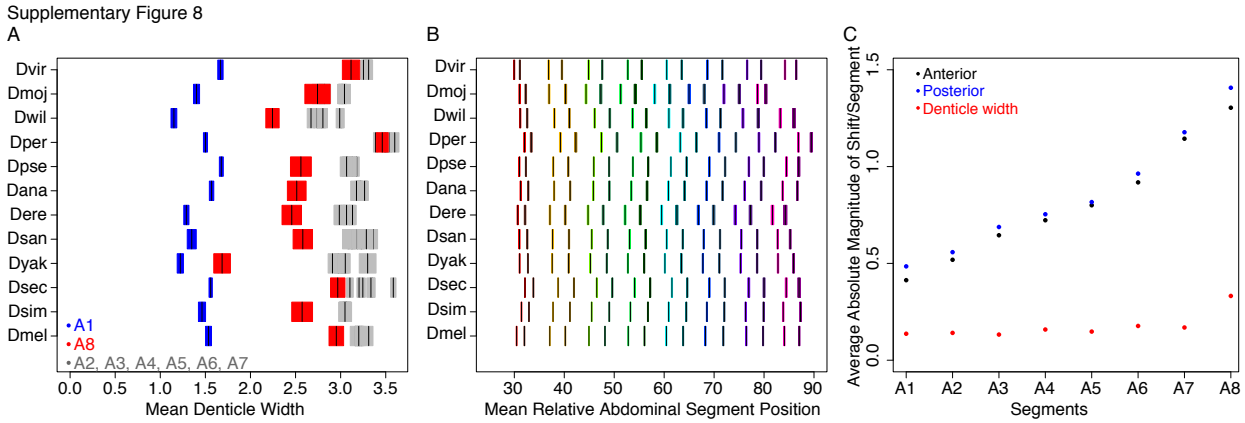


Supplementary Figure 6. These graphs have the same format as Figure 2B and shows differences in relative segment position between species. (A) Relative segment positions are calculated in the absence of A8+tail. Notice a substantial number of nonsignificant differences as compared Figure 2B. (B) Relative segment positions are calculated in the absence of h+t and also with measurements taken from the posterior of the larva instead of the anterior. As compared to Figure 2B the segment order is flipped, but the pattern of change in segment position is very similar. In both graphs, each black bar depicts the mean for each segment in each species. The colored areas are 95% confidence intervals. The y axis depicts 12 species and x axis shows mean relative abdominal segment position.

Supplementary Figure 7

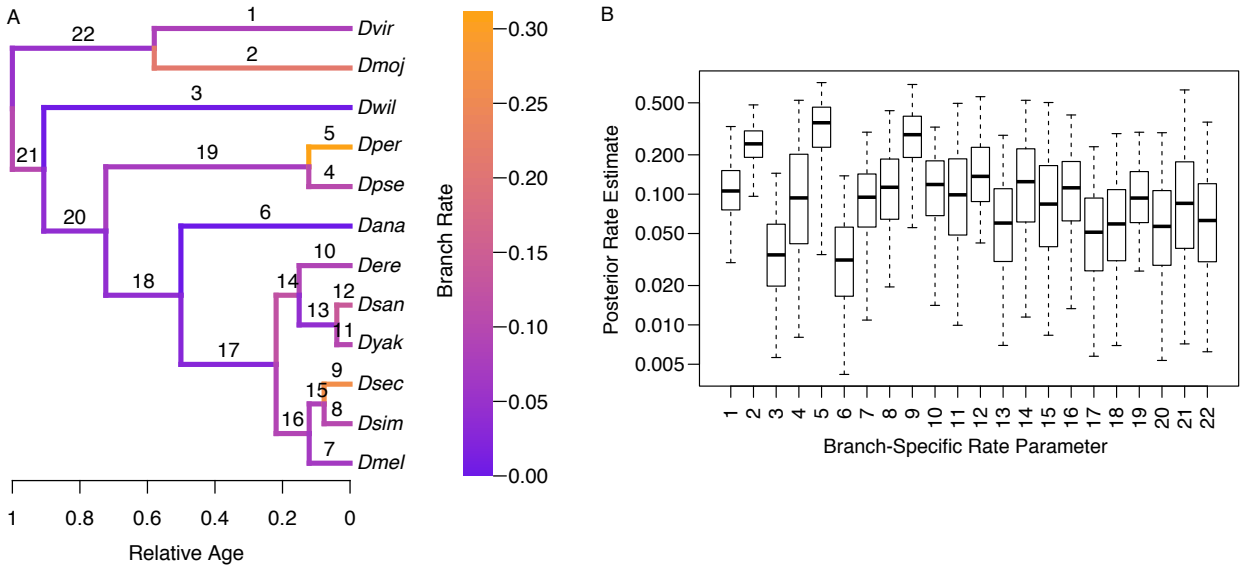


Supplementary Figure 7. (A) Change in coefficient of variation, averaged over 12 species, from anterior to posterior segments. The black dots represent the case when relative segment positions were calculated with all segments included. The y axis shows coefficient of variation and the y axis depicts each abdominal segment. (B) Change in standard deviation, averaged over 12 species, from anterior to posterior segments. The black dots represent the case when relative segment positions were calculated with all segments included. The y axis shows coefficient of variation and the y axis depicts each abdominal segment. In both graphs, the red and blue dots represent the cases when relative segment positions were calculated in the absence of A8+tail or h+t, respectively.



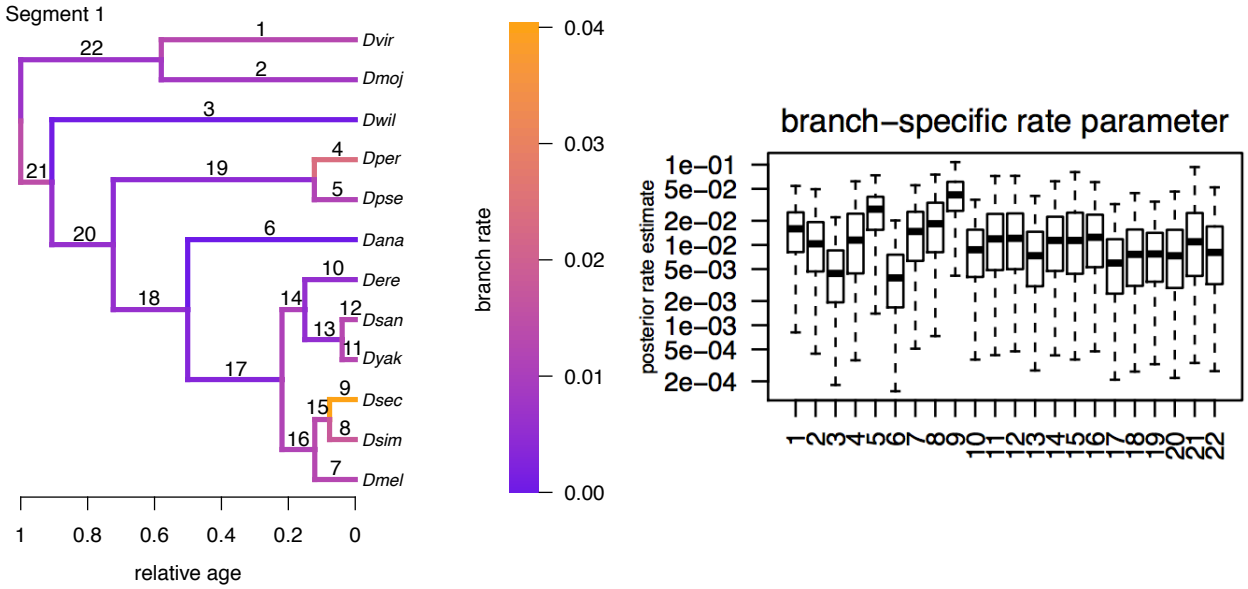
Supplementary Figure 8. (A) This graph shows differences in denticle width between 12 species. Blue represents segment A1, red represents segment A8 and gray represents the rest of the segments. Black bars represent the mean and the colored area around black bars are 95% confidence intervals. The y axis in this graph depicts 12 Drosophila species, the x axis shows mean denticle width. (B) This graph has the same format as Figure 2B, however in addition to differences in relative segment position (anterior border of denticle belts) it also shows changes in the posterior border of abdominal denticle belts between 12 species. The y axis depicts 12 species and x-axis shows mean relative abdominal segment position. Bars showing changes in posterior border of denticle belts are shaded darker than bars representing changes in the anterior borders. (C) This graph shows deviation from mean, averaged over all species, for the anterior (black), posterior (blue) and width (red) of denticle belts for each abdominal segment. The y axis show deviation from mean and the x axis depicts each abdominal segment.

Supplementary Figure 9

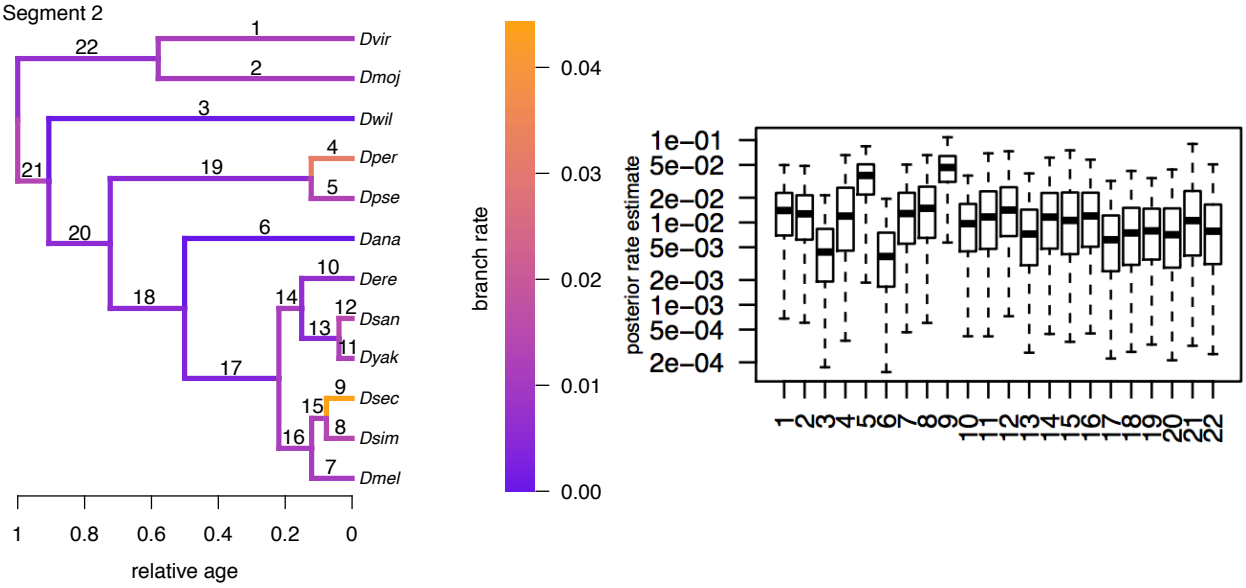


Supplementary Figure 9. Phylogenetic analysis of segment evolution in the 12 *Drosophila* species, with posterior rate estimates for all branches. A) Like Figure 4A, the phylogeny was inferred from nuclear loci with relative divergence times, and branches of the tree are colored to indicate the overall rate of relative segment position evolution. Here, the branches are numbered to correspond with the values pictured in part B. B) Box plot depicting rate of evolution estimate for each branch of the phylogeny, x-axis corresponds to the branch numbers indicated on the tree in part A.

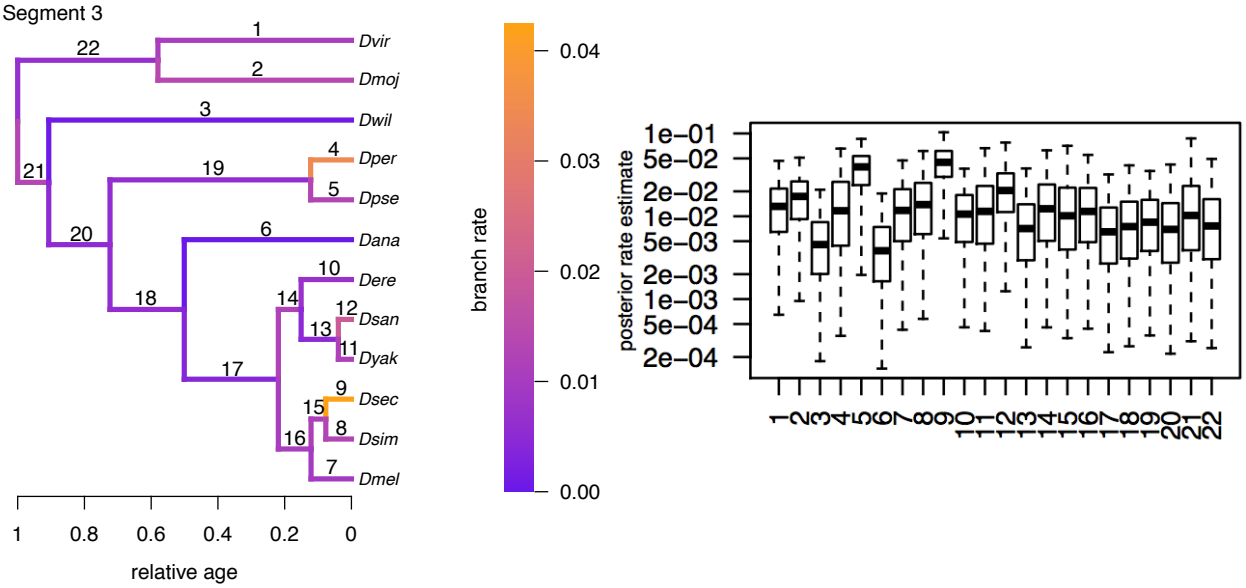
Supplementary Figure 10



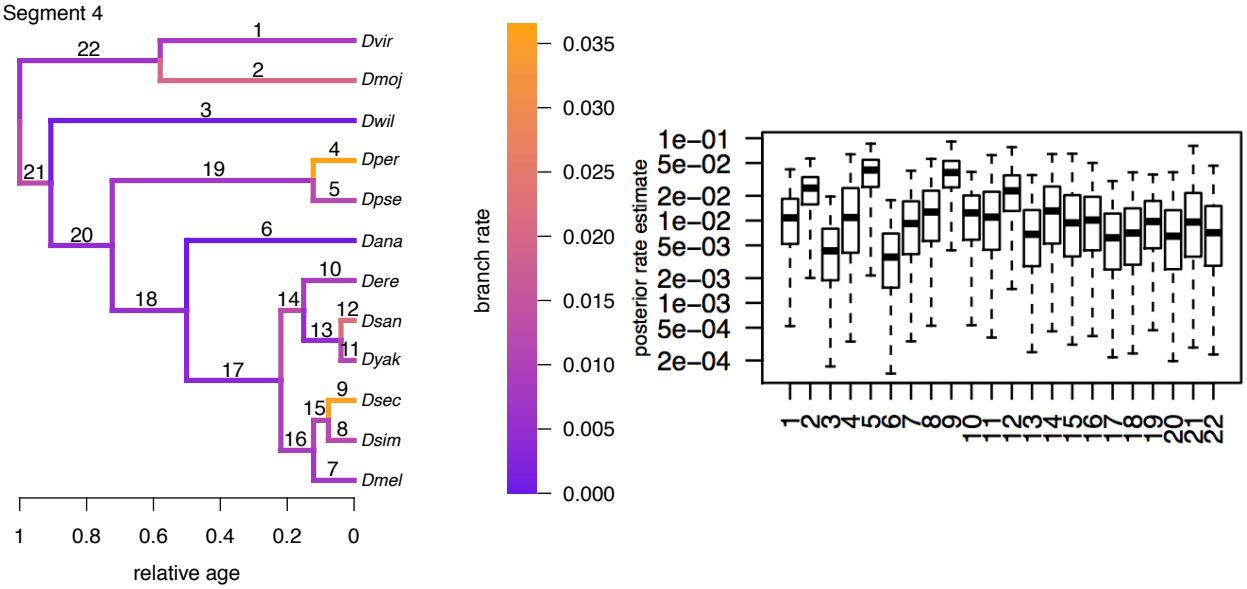
Supplementary Figure 10
Segment 2



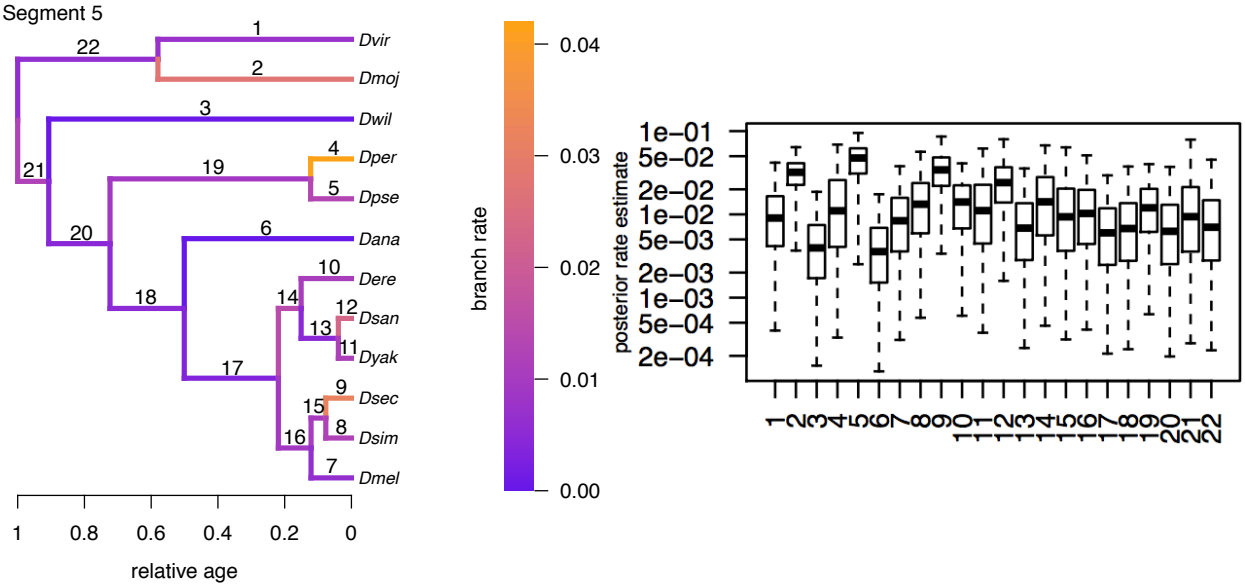
Supplementary Figure 10
Segment 3



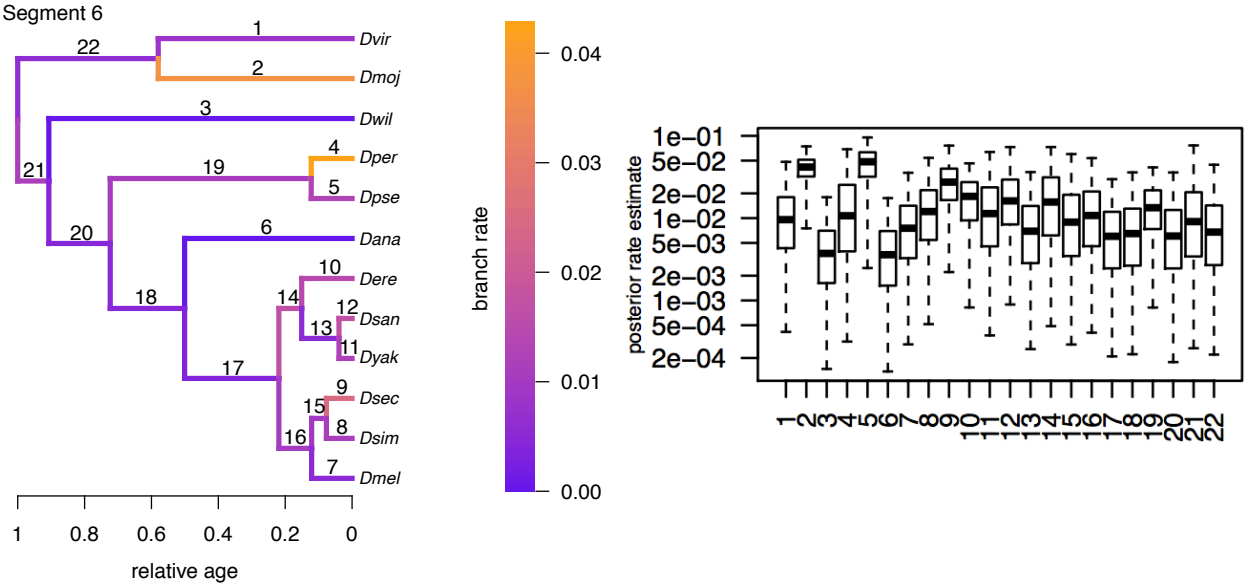
Supplementary Figure 10
Segment 4



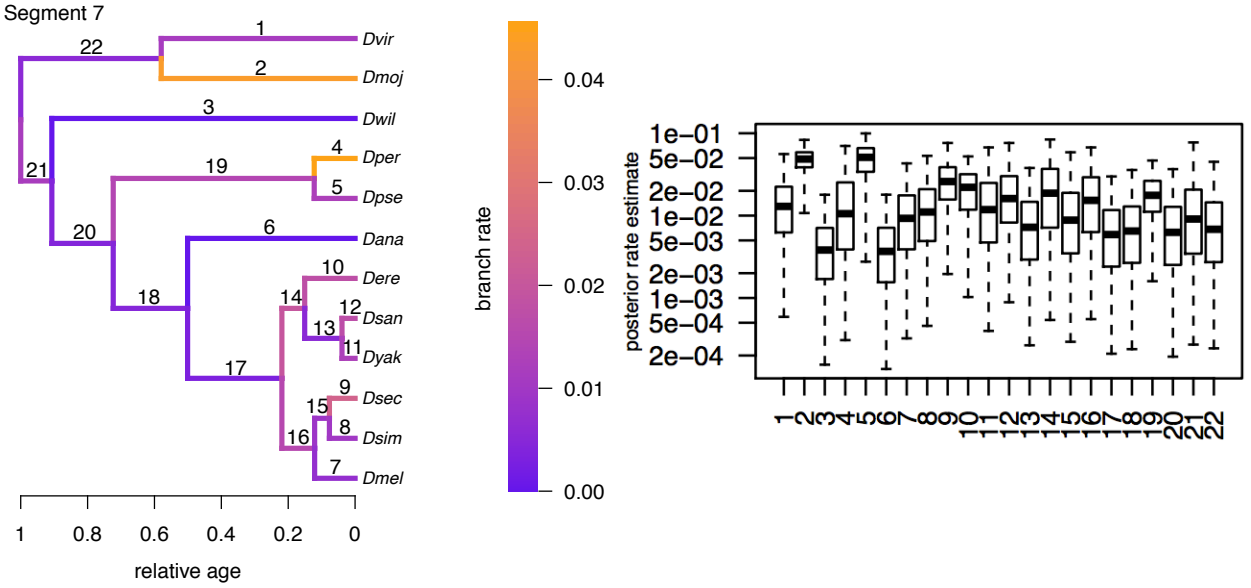
Supplementary Figure 10
Segment 5

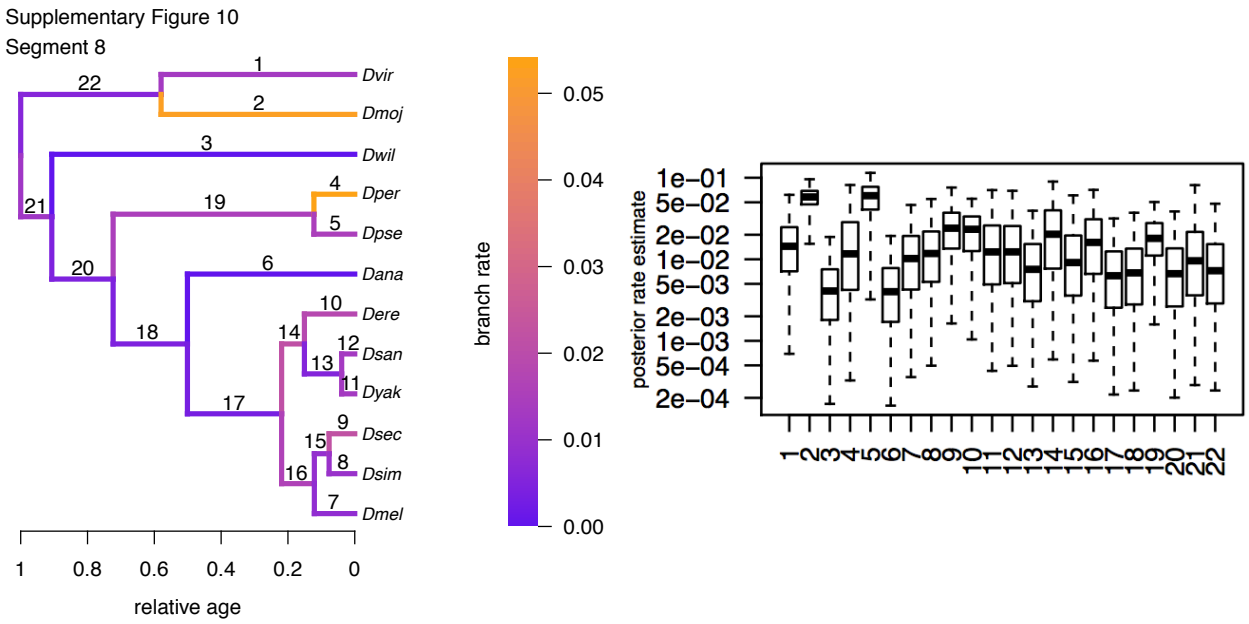


Supplementary Figure 10
Segment 6



Supplementary Figure 10
Segment 7

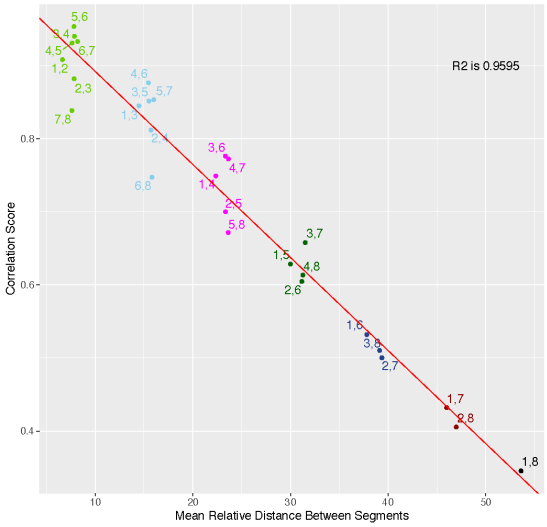




Supplementary Figure 10. Phylogenetic analysis of relative segment position for each segment. Like Figure 4A, this figure shows a phylogeny inferred from nuclear loci with relative divergence times, and the branches of the tree are colored to indicate the overall rate of segment position evolution. Like Supplementary Figure 9, the tree diagrams also include numbers for each linage, and are accompanied by a box plot showing the estimates for rates of segment evolution by each numbered lineage. Here, there are 8 plots, to show rate estimates for each abdominal segment separately.

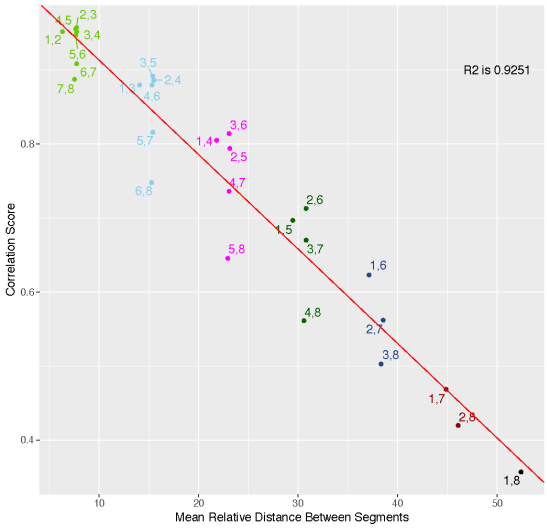
Supplementary Figure 11

D.melanogaster Correlation score vs Pairwise distance



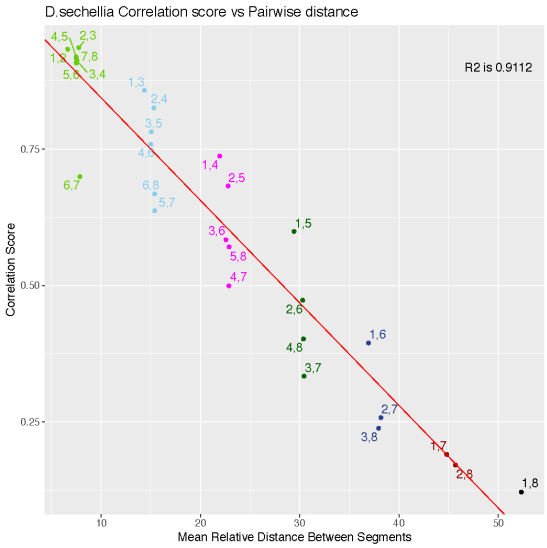
Supplementary Figure 11

D.simulans Correlation score vs Pairwise distance



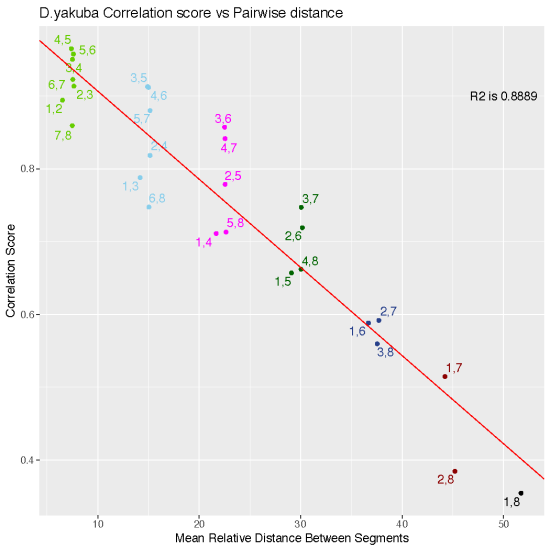
Supplementary Figure 11

D.sechellia Correlation score vs Pairwise distance



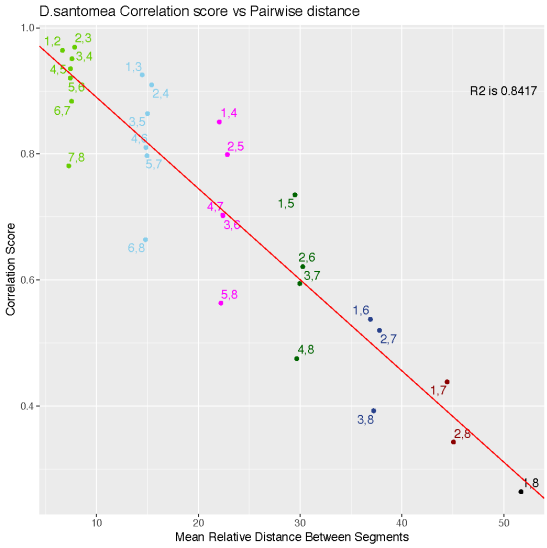
Supplementary Figure 11

D.yakuba Correlation score vs Pairwise distance



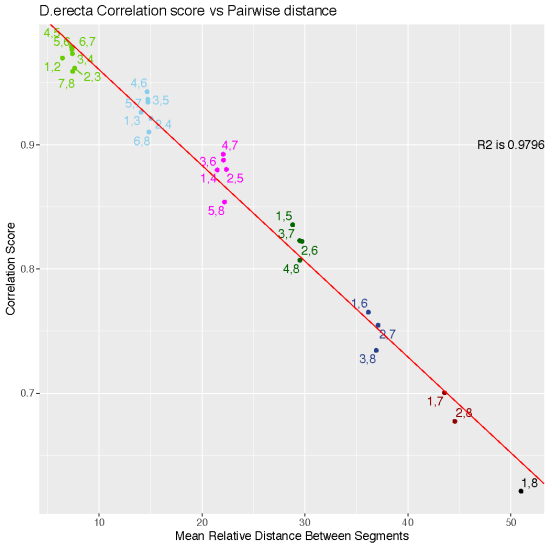
Supplementary Figure 11

D.santomea Correlation score vs Pairwise distance



Supplementary Figure 11

D.erecta Correlation score vs Pairwise distance



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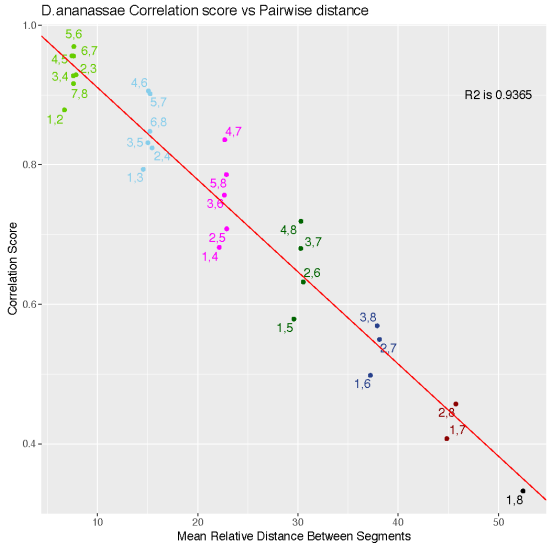
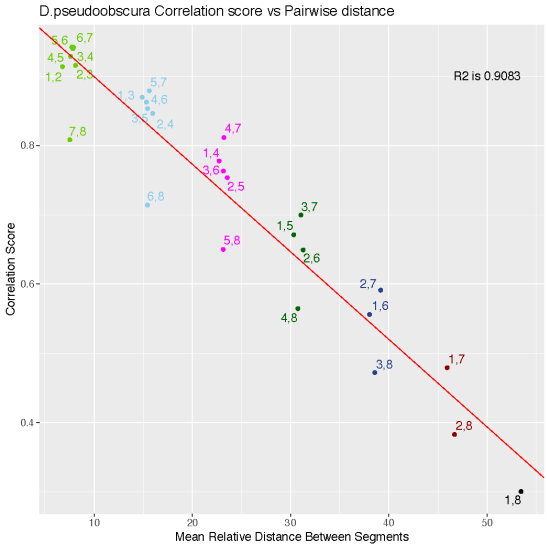
Supplementary Figure 11

D.ananassae Correlation score vs Pairwise distance



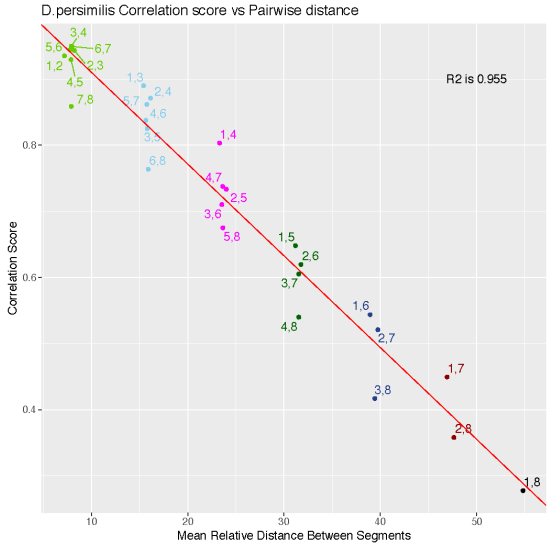
Supplementary Figure 11

D.pseudoobscura Correlation score vs Pairwise distance



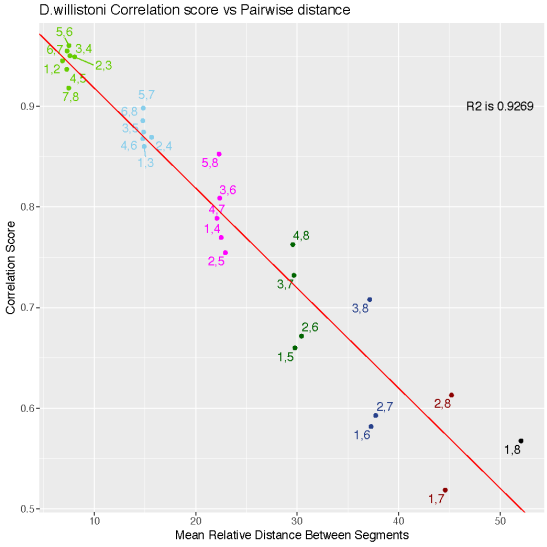
Supplementary Figure 11

D.persimilis Correlation score vs Pairwise distance



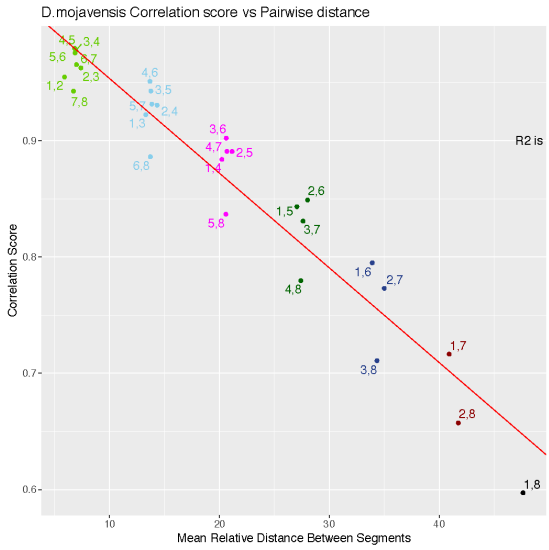
Supplementary Figure 11

D.willistoni Correlation score vs Pairwise distance



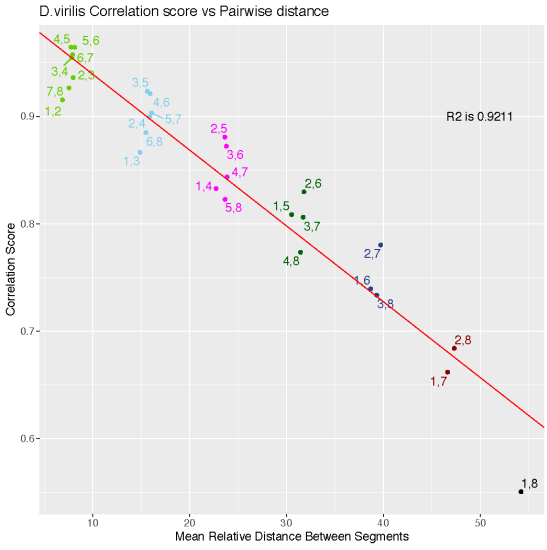
Supplementary Figure 11

D.mojavensis Correlation score vs Pairwise distance



Supplementary Figure 11

D.virilis Correlation score vs Pairwise distance

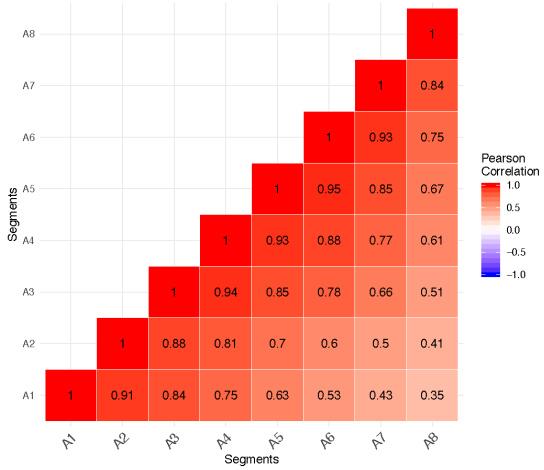


1106

1107 **Supplementary Figure 11.** This series of graphs are constructed the same as Figure
1108 6A, which was for *D. melanogaster*, for each of the 12 Drosophila species in our study.
1109 The y axis shows correlation scores and the x axis shows mean relative distance
1110 between pairs of segments.
1111
1112

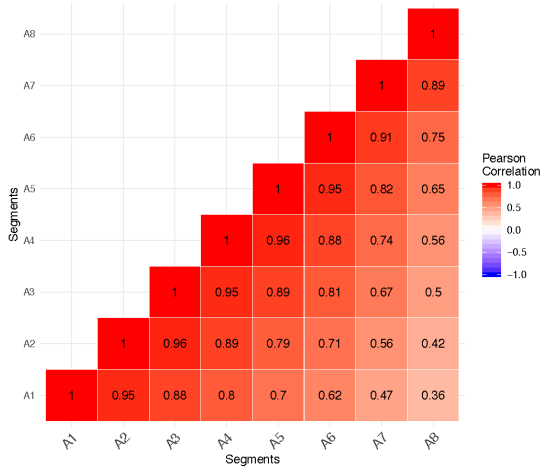
Supplementary Figure 12

D.melanogaster Correlation Score Heatmap



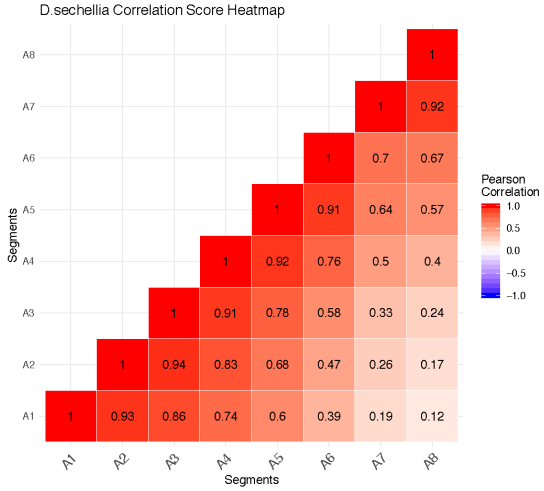
Supplementary Figure 12

D.simulans Correlation Score Heatmap



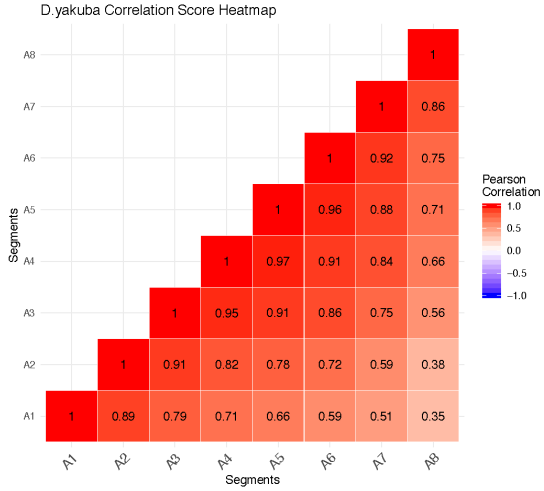
Supplementary Figure 12

D.sechellia Correlation Score Heatmap



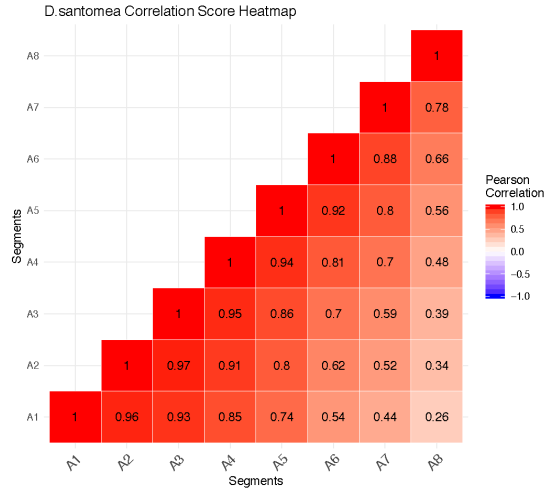
Supplementary Figure 12

D.yakuba Correlation Score Heatmap



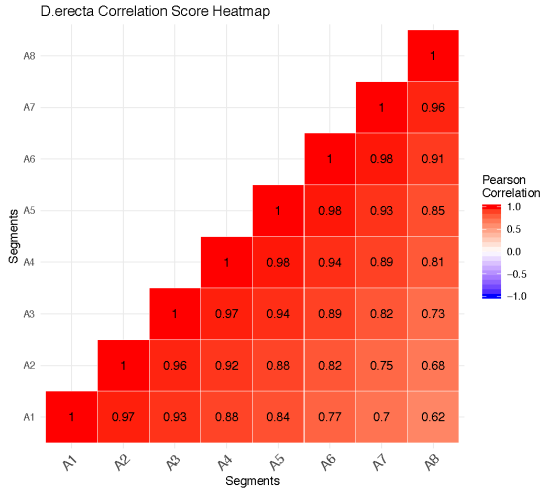
Supplementary Figure 12

D.santomea Correlation Score Heatmap



Supplementary Figure 12

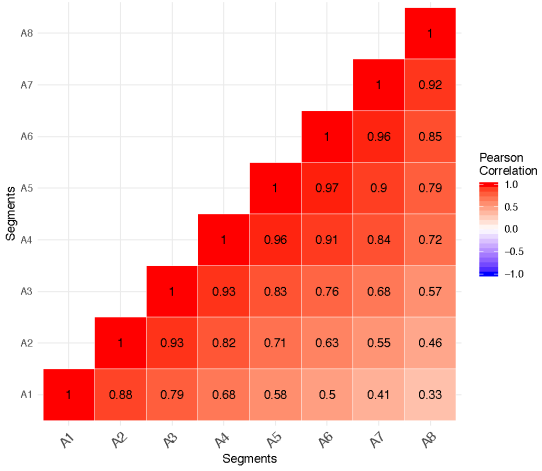
D.erecta Correlation Score Heatmap



1113

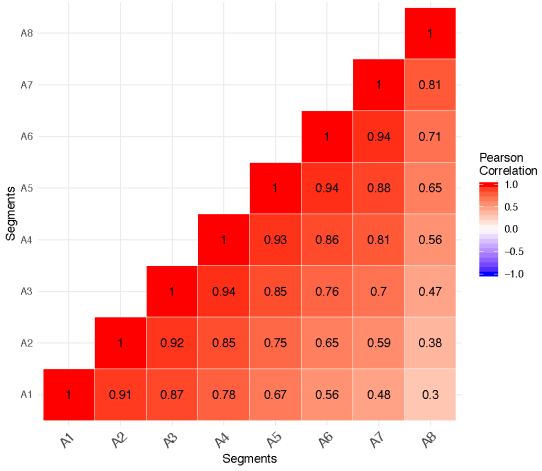
Supplementary Figure 12

D.ananassae Correlation Score Heatmap



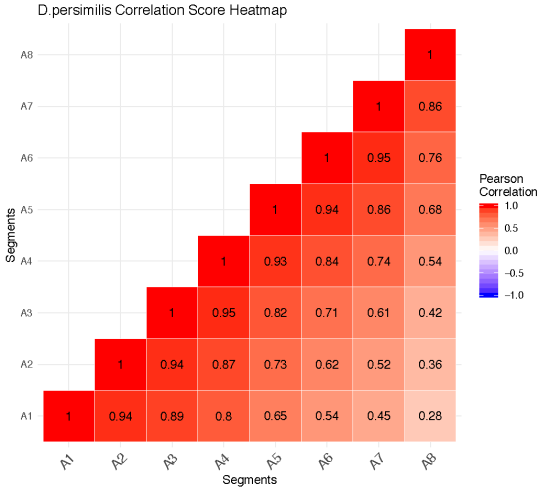
Supplementary Figure 12

D.pseudoobscura Correlation Score Heatmap



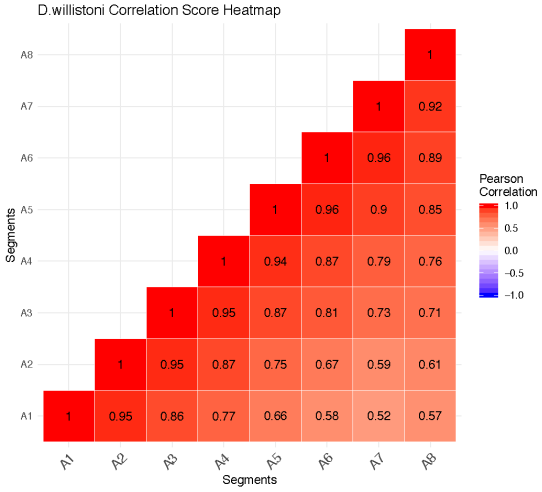
Supplementary Figure 12

D.persimilis Correlation Score Heatmap



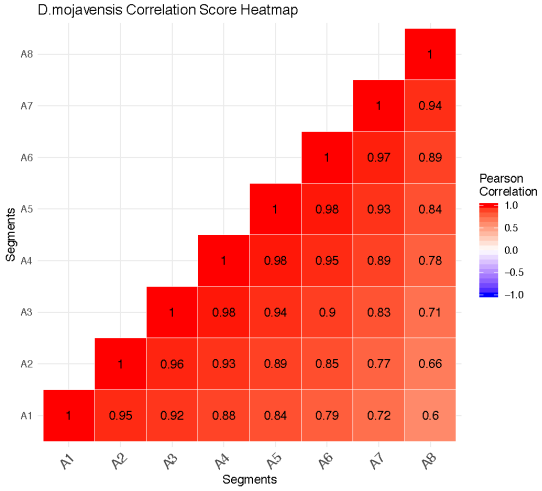
Supplementary Figure 12

D.willistoni Correlation Score Heatmap



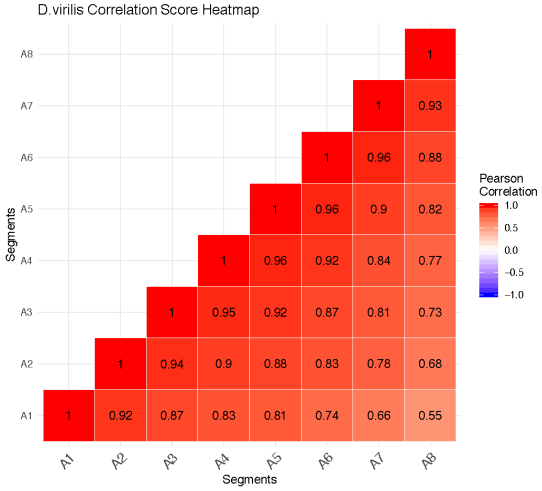
Supplementary Figure 12

D.mojavensis Correlation Score Heatmap



Supplementary Figure 12

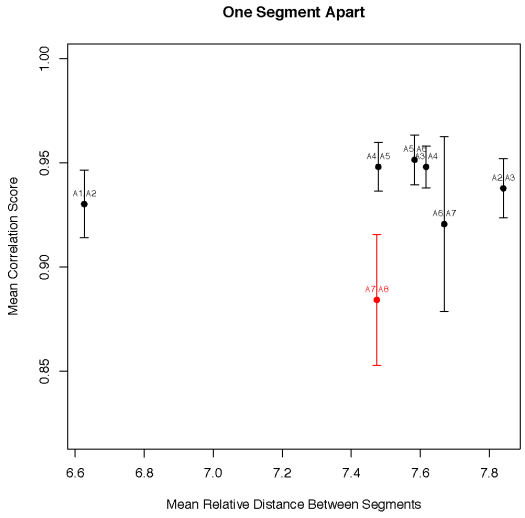
D.virilis Correlation Score Heatmap



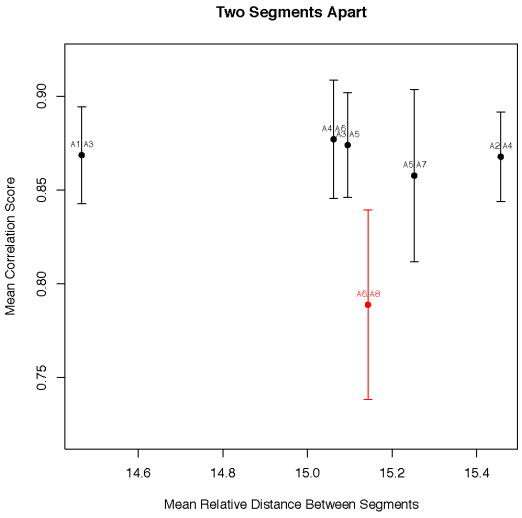
1114

Supplementary Figure 12. Series of correlation score heat maps for each of the 12 species in our study. There is a square showing the correlation score for the shift in the relative position of each segment pair within a species. Red means high positive correlation and white means low positive correlation. Actual correlation scores are printed on each square box. Notice that for most species, the color scheme is lighter at the right edge of the triangle as compared to the bottom edge of the triangle. This is consistent with other figures showing shifts in the position of A8 has worse correlation with shifts in the position of other segments, regardless of the distance in between.

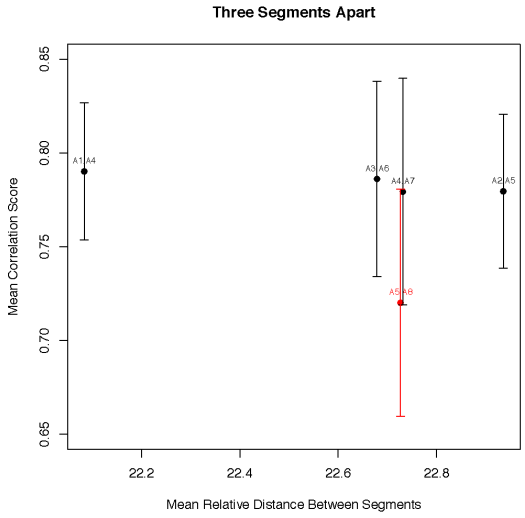
Supplementary Figure 13



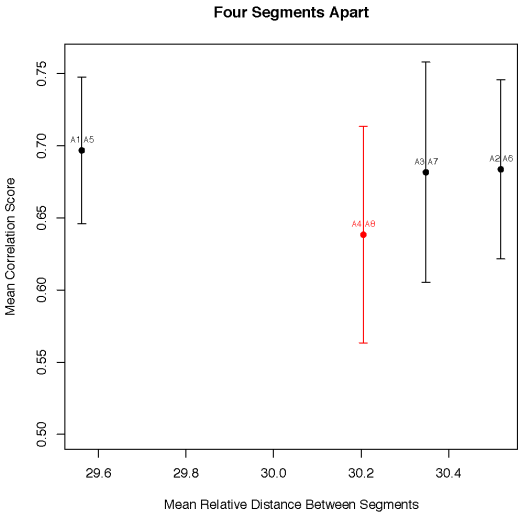
Supplementary Figure 13



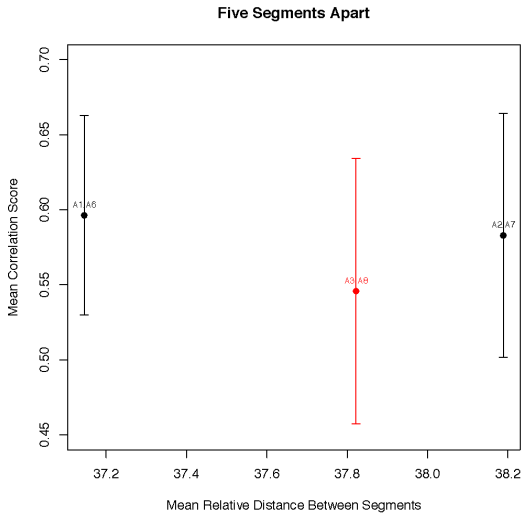
Supplementary Figure 13



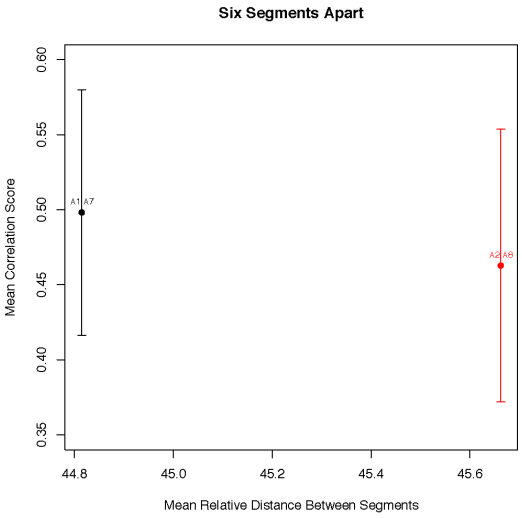
Supplementary Figure 13



Supplementary Figure 13



Supplementary Figure 13

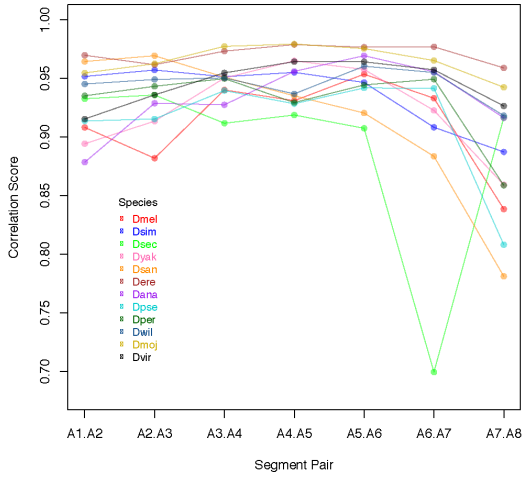


1125

Supplementary Figure 13. This series of graphs represent the information from Figure 6B, in a zoomed in format. Each figure has mean correlation score over all species on the y axis and mean relative distance over all species on the x axis. Segments that are paired with A8 are in red. Error bars are 95% confidence intervals.

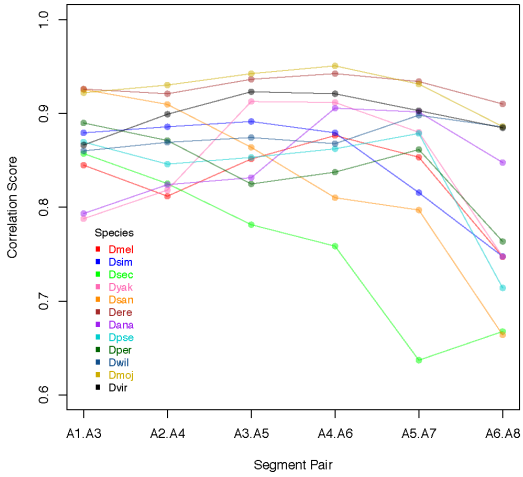
Supplementary Figure 14

Correlation Score vs Segment Pair – One



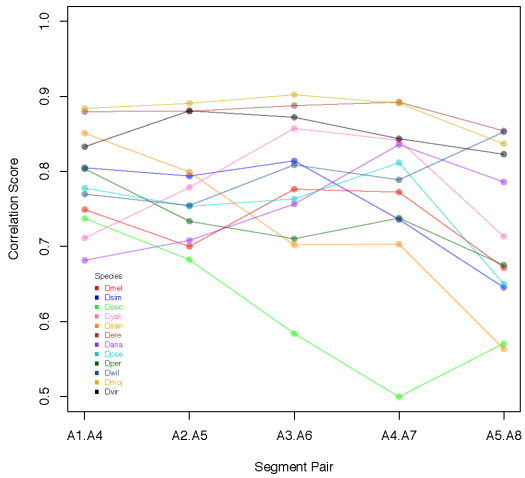
Supplementary Figure 14

Correlation Score vs Segment Pair – Two



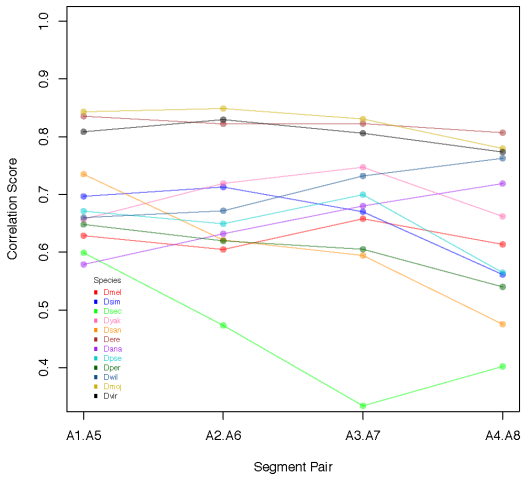
Supplementary Figure 14

Correlation Score vs Segment Pair – Three



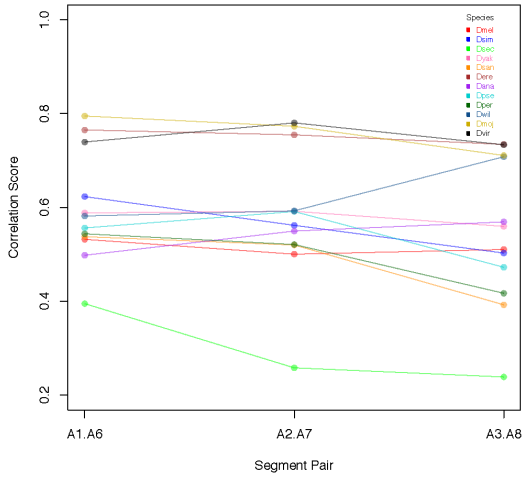
Supplementary Figure 14

Correlation Score vs Segment Pair – Four



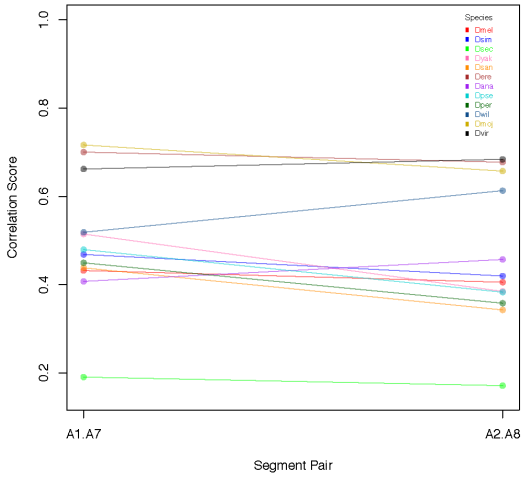
Supplementary Figure 14

Correlation Score vs Segment Pair – Five



Supplementary Figure 14

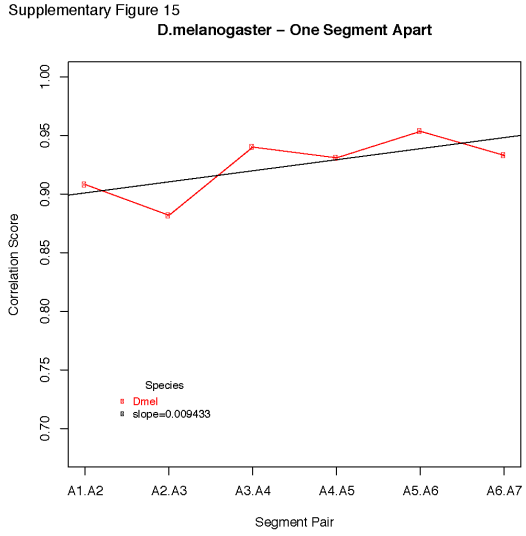
Correlation Score vs Segment Pair – Six



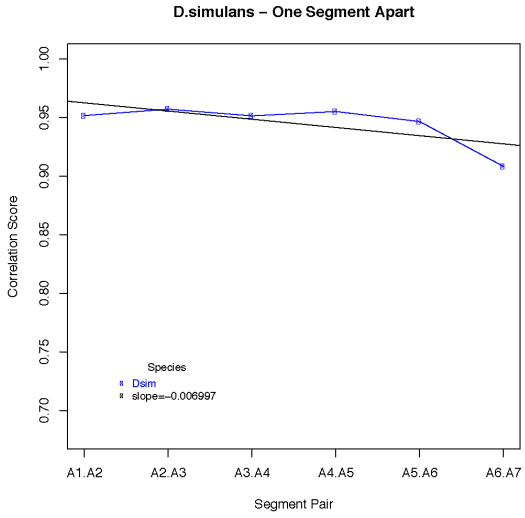
1132

Supplementary Figure 14. This series of graphs show how correlation scores change from anterior to posterior in all species. In each graph in the series, segment pairs become further apart from each other (from one to seven segments apart). y axis represents correlation scores and the x axis represents each of the eight abdominal segments. Each species is represented by a different color. Notice, especially as the pairs of segments become further apart, *D. erecta*, *D. mojavensis* and also *D. virilis* stand out as the species with the highest correlation scores.

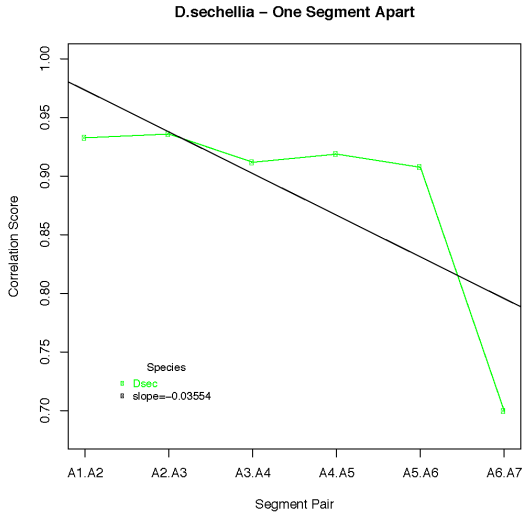
Supplementary Figure 15



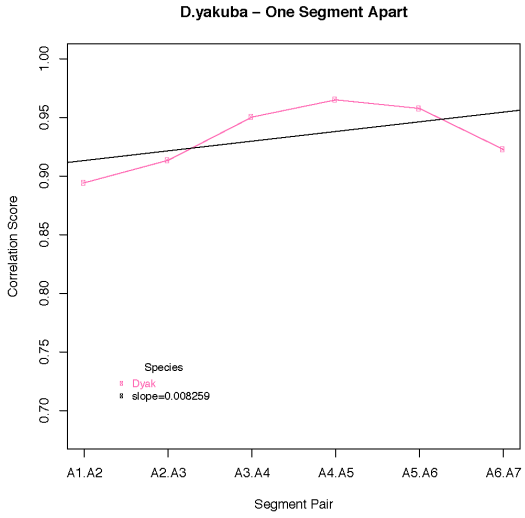
Supplementary Figure 15



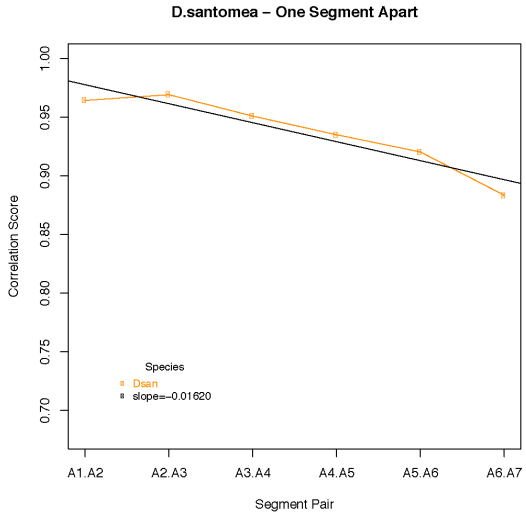
Supplementary Figure 15



Supplementary Figure 15



Supplementary Figure 15



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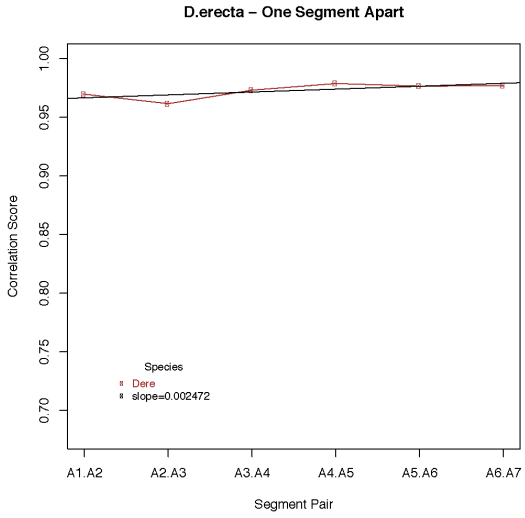
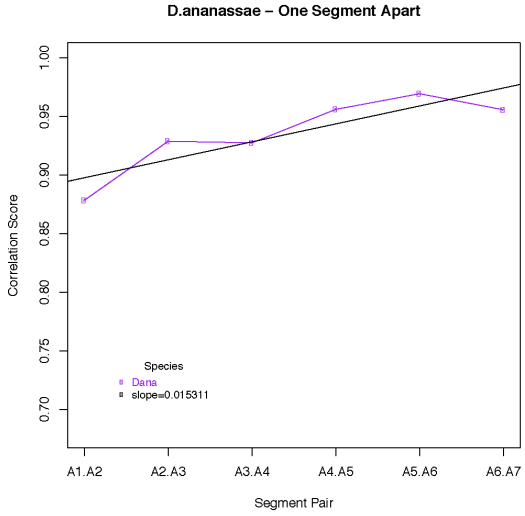
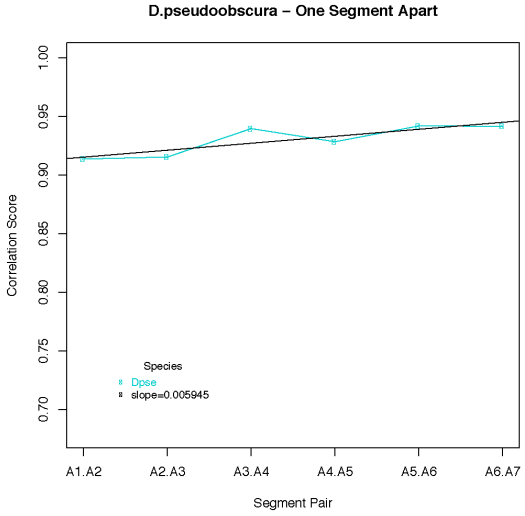


1142

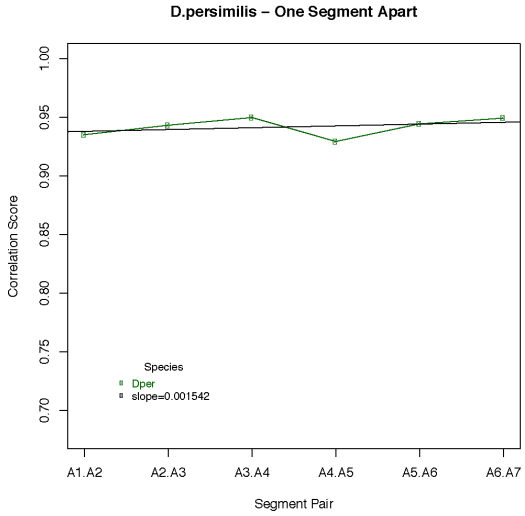
Supplementary Figure 15



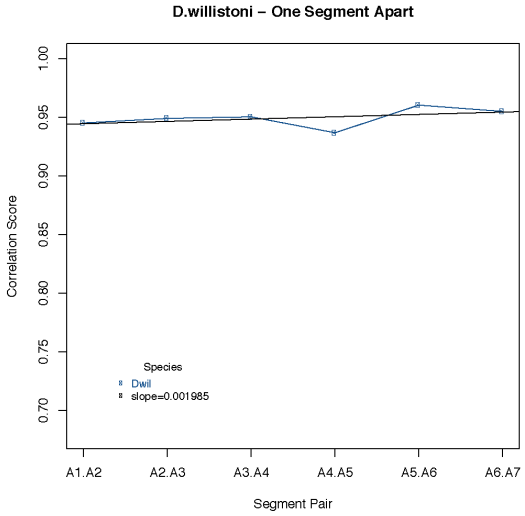
Supplementary Figure 15



Supplementary Figure 15



Supplementary Figure 15



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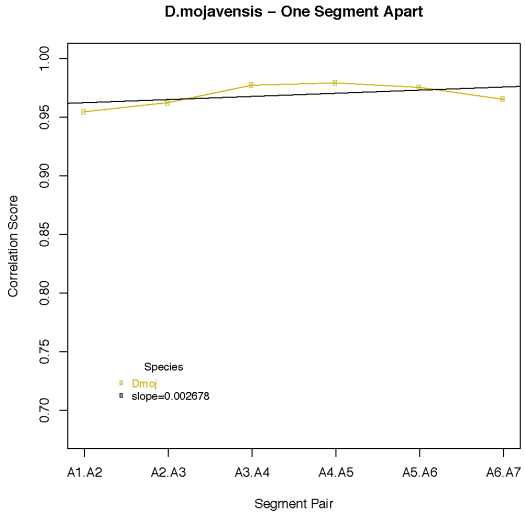
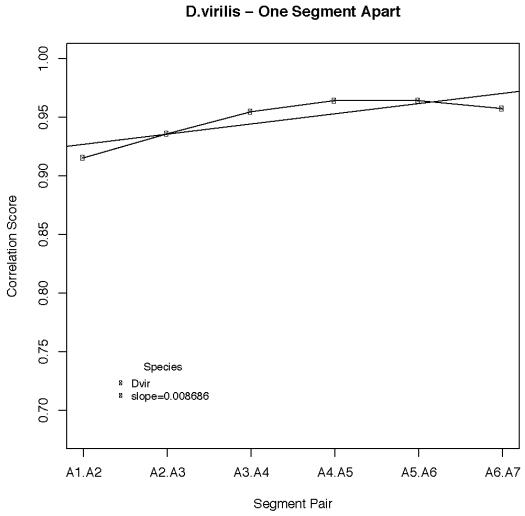


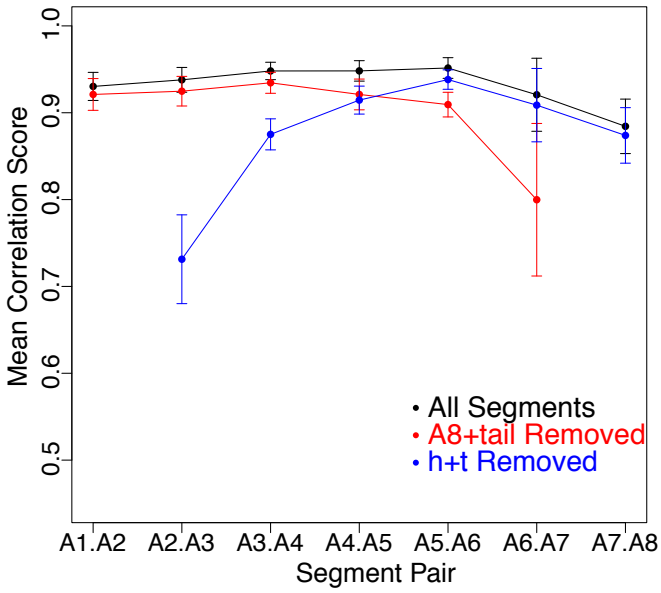
Supplementary Figure 15



1143

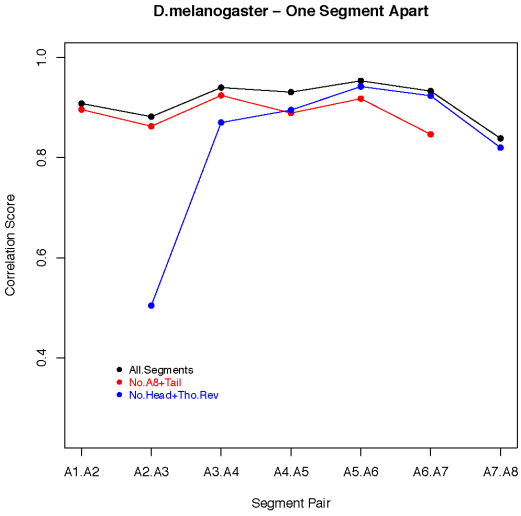
Supplementary Figure 15. This series of graphs show how correlation scores change for each species from anterior to posterior. It is essentially a broken down version of the first graph in Supplementary Figure 12, without “A7.A8”. Slope for each trend line is reported in the legend to give an idea of direction of change in correlation scores.

Supplementary Figure 16

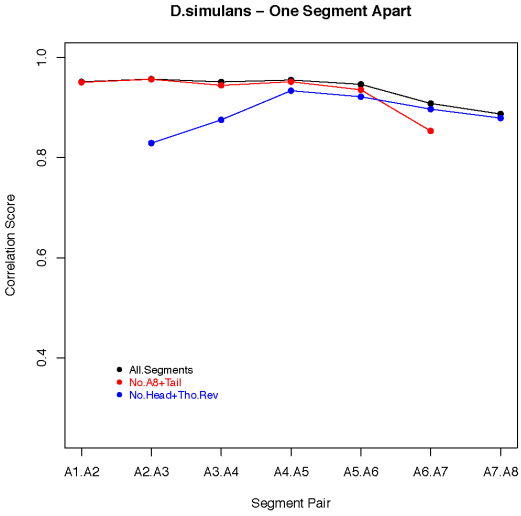


Supplementary Figure 16. When all segments are included in position calculations, mean correlation scores between position shifts of segments that are next to each other are highest ever so slightly in the middle of the larva. In comparison, when A8+tail is removed from position calculations, correlation between segment position shifts at the posterior of the larvae goes down significantly. This is true for the anterior part of the larva when h+t are removed from position calculations.

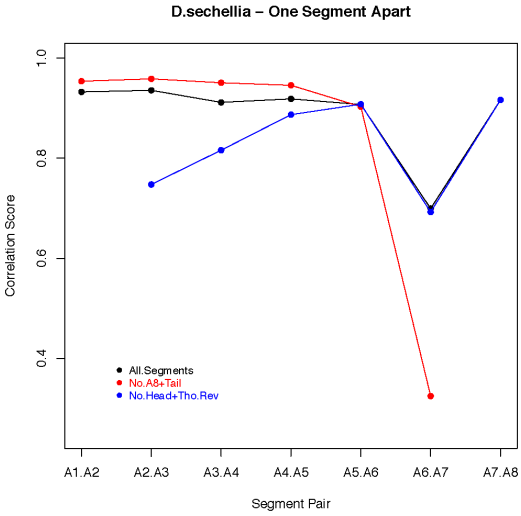
Supplementary Figure 17



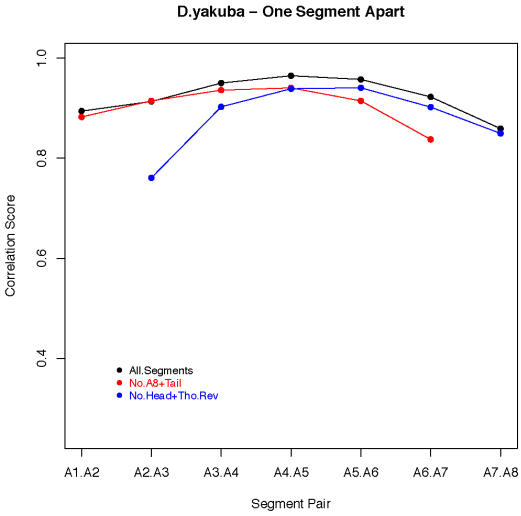
Supplementary Figure 17



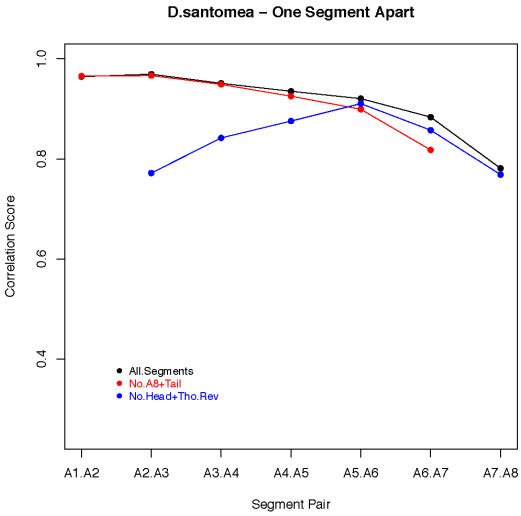
Supplementary Figure 17



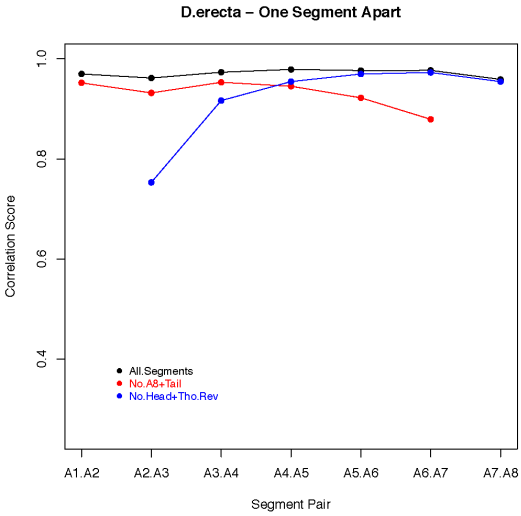
Supplementary Figure 17



Supplementary Figure 17



Supplementary Figure 17



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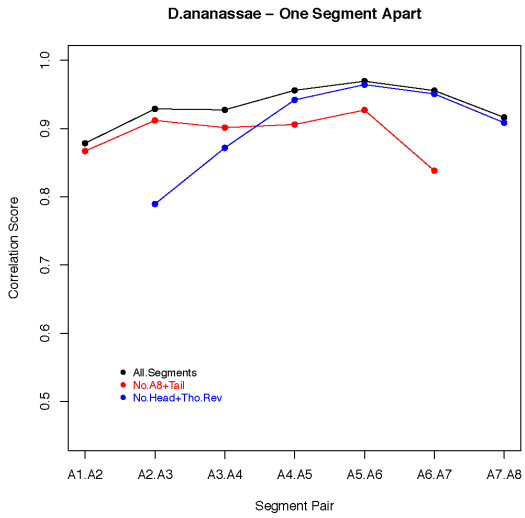
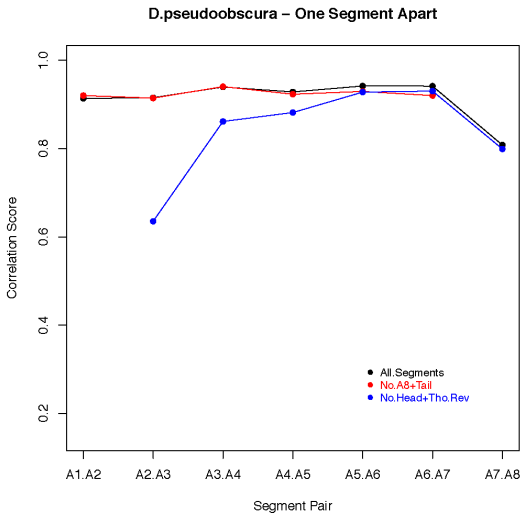
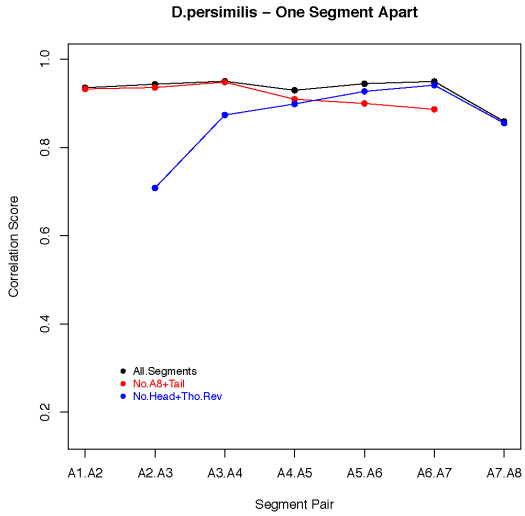
Supplementary Figure 17



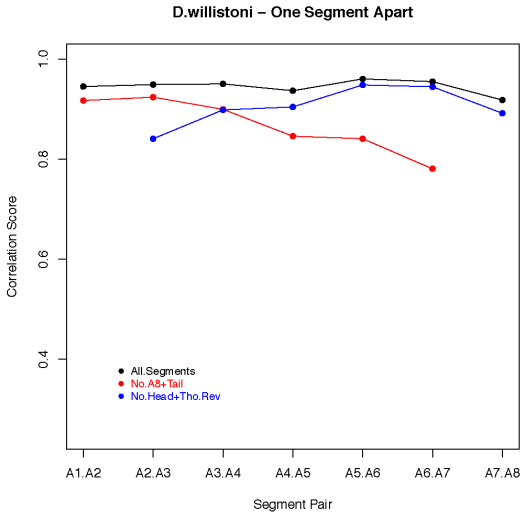
Supplementary Figure 17



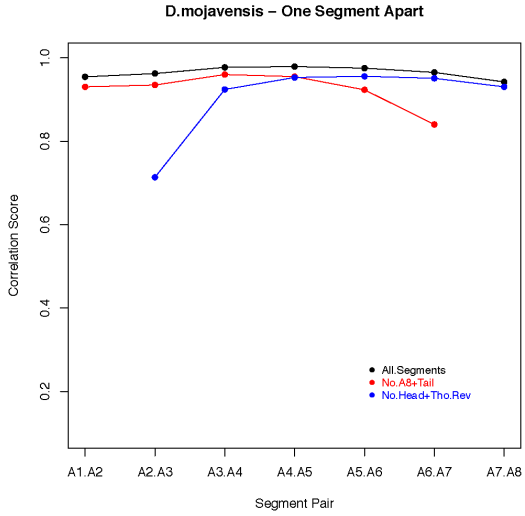
Supplementary Figure 17



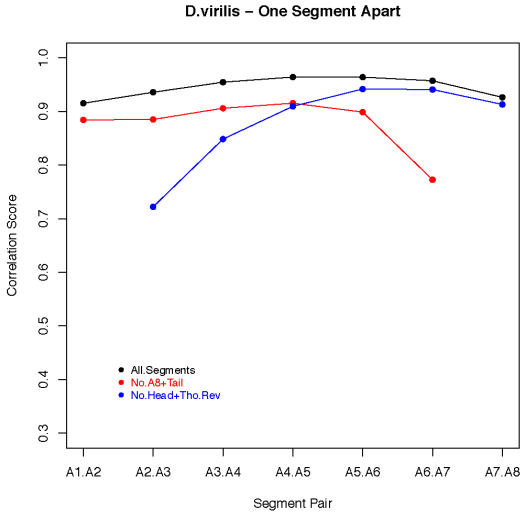
Supplementary Figure 17



Supplementary Figure 17



Supplementary Figure 17



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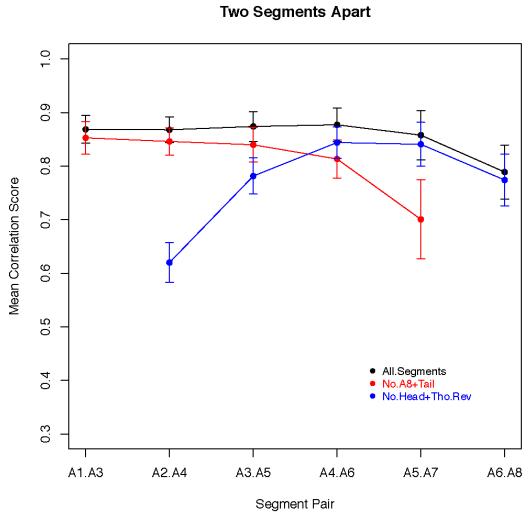
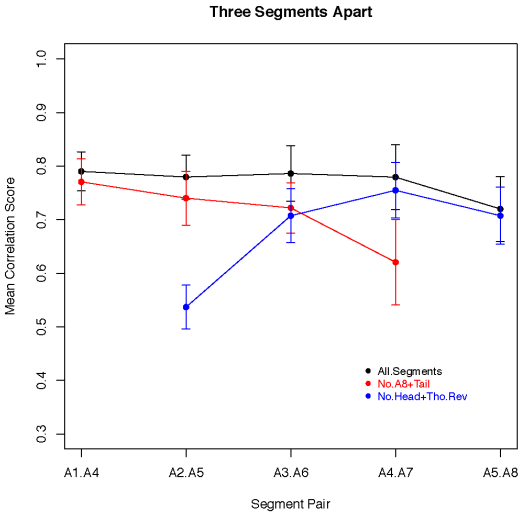
Supplementary Figure 17. This series of graphs are a broken-down version of Supplementary Figure 16 into each species. They show, for each species, how correlation scores change from the anterior to the posterior of the larvae, when relative segment position was calculated with all segments included (black) versus no A8+tail (red) or no h+t (blue).

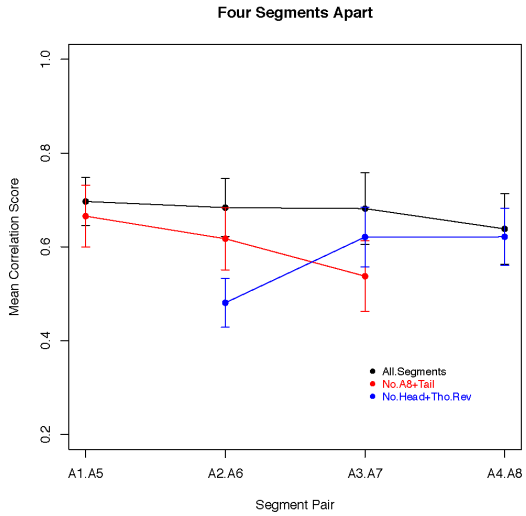
Supplementary Figure 18

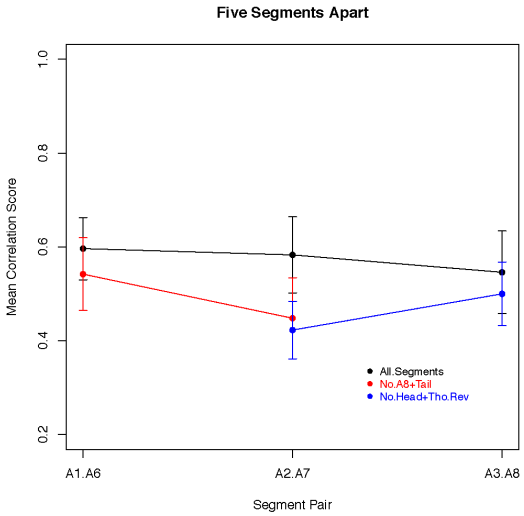


Supplementary Figure 18

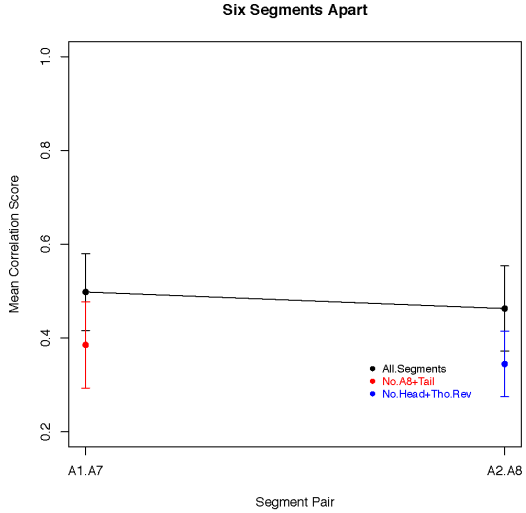


Supplementary Figure 18





Supplementary Figure 18



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Supplementary Figure 18. This series of graphs are a continuation of Supplementary Figure 16, as the distance between pairs of segments increase, from two to six segments apart. Notice that removal of h+t overall decreases correlation scores more than removal of A8+tail.

Supplementary Table 1. Empirical data collected in the Lott Lab on number of flies and days needed in a bottle to control population density of each species. Also includes, for each species, approximate embryonic developmental duration at 20°C and number of minutes needed in the 60°C oven for larvae to become straight.

Species Name	Number of Male and Female flies per bottle	Number of days in bottle before removing adults	Number of days from egg to larva at 20°C (hours)	Number of minutes at 60° oven
D. melanogaster (Ore	15 each	4-5 days	~36	50
D. simulans	15 each	4-5 days	~24	50
D. sechellia	20 each	7 days	~36	90
D. yakuba	10 each	4-5 days	~24	50
D. santomea	25 each	7 days	~36	50
D. erecta	10 each	4-5 days	~24	50
D. ananassae	20 each	7 days	~36	50
D. pseudoobscura	10 each	7days	~36	50
D. persimilis	15 each	7 days	~36	50
D. willistoni	25 each	7 days	~48	50
D. mojavensis	25 each	7 days	~72	45
D. virilis	15 each	7 days	~60	90

Supplementary Table 2. Lists the number of segments that are differentially positioned between pairs of species

Supplementary Table 3. Lists the deviation of the position of each segment in each species from the “across-species” mean.

Supplementary File 1. Methods and results of the phylogenetic analysis.

Supplementary File 2. Additional information on the observed changes in the position of posterior border of denticle belts and denticle width between species.

Supplementary File 3. Description of the effects of removal of A8+tail or h+t from segment position calculations. In particular, effects of the new calculations on differences in segment position between species as well as the noise in segment position within a species.