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 across the phylogeny. 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

43 the length of the embryo (St Johnston and Nüsslein-Volhard 1992; Surkova et al. 2018).
44 These maternal gene products regulate one another, and also regulate genes
45 expressed later in development by the zygote (St Johnston and Nüsslein-Volhard 1992;
46 Surkova et al. 2018). This genetic network of regulators, which has been well-
47 established through decades of critical study (Nüsslein-Volhard and Wieschaus 1980;
48 Kornberg and Tabata 1993; Nasiadka et al. 2002; Nüsslein-Volhard et al. 2008; Clark
49 2017) precisely divide the embryo into progressively smaller subsections over
50 embryonic development, until the correct number of body segments is reached. At the
51 end of the embryonic stage, a first-instar larva with a highly organized segmented
52 pattern of differentiated structures is produced.

53 As long as segmentation has been investigated, its fundamental role in
54 *Drosophila* development has been clear, as defects in this process can be detrimental
55 (or lethal) to the organism (Wieschaus and Nuesslein-Volhard 2016). Indeed, the
56 lethality of homozygous mutations in segmentation genes was critical to their discovery
57 in mutant screens (Wieschaus and Nuesslein-Volhard 2016). Subsequent generations
58 of experiments using increasingly advanced methods have demonstrated that in
59 addition to being critical, segmentation is a very precise process, with measurements of
60 expression domains of segmentation genes being highly reproducible between embryos
61 (Houchmandzadeh et al. 2002; Gregor et al. 2007; Surkova et al. 2008; Jaeger 2010;
62 Petkova et al. 2014; Bentovim et al. 2017). Additionally, research has shown that
63 segmentation can proceed correctly, while keeping its precision, in the face of
64 substantial perturbations. For instance, in a seminal study launching the interface

65 between the gradient of the maternal gene *bicoid (bcd)* and its downstream target
66 *hunchback (hb)* as a model for understanding precision and scaling in developmental
67 signaling, Houchmandzadeh et al. (2002), also demonstrated that the relative position of
68 the Hb expression boundary (relative to embryo size) was robust to substantial genetic
69 and environmental perturbations. This study tested genetic perturbations in the form of
70 mutations in important maternal genes or absence of whole chromosomes, and found
71 very little or no variation in the relative position of Hb expression (Houchmandzadeh et
72 al. 2002). Moreover, the standard deviation of the Hb expression boundary did not show
73 a significant increase with these drastic perturbations, indicating that these genetic
74 changes did not decrease precision in the Hb boundary (Houchmandzadeh et al. 2002).
75 There is also substantial evidence that segmentation proceeds precisely in the face of
76 dramatic environmental perturbation. For example, when whole (Houchmandzadeh et
77 al. 2002) or two halves (Lucchetta et al. 2005) of *D. melanogaster* embryos were raised
78 at different temperature extremes after fertilization, inducing different developmental
79 rates, expression domains were formed in the same relative position and with the same
80 level of precision observed in control embryos, by the gap or pair rule stage of
81 embryonic development, respectively (Houchmandzadeh et al. 2002; Lucchetta et al.
82 2005).

83 The above studies, along with many others, show that *Drosophila* segmentation
84 is a complex trait that is robust to many forms of variation (Driever and Nüsslein-Volhard
85 1988; Houchmandzadeh et al. 2002; Lucchetta et al. 2005; Manu et al. 2009b). It is
86 perhaps unsurprising then that with the exception of some Hawaiian *Drosophila* species

87 (Spieth 1981), it is difficult to observe any gross differences in segment position, size
88 and shape, scaled to full body size, between adults of different *Drosophila* species.
89 However, over longer evolutionary time scales, segment patterning can vary
90 substantially between arthropod species (Regier et al. 2010). At the early embryonic
91 stage in *Drosophila*, previous studies have shown small quantitative differences in
92 relative position of segmentation gene expression domains between both closely related
93 (Lott et al. 2007; 2010; Fowlkes et al. 2011; Combs and Fraser 2018) and more
94 distantly related species (Fowlkes et al. 2011; Wunderlich et al. 2019). Within a species,
95 there is little evidence for variation in the relative position of segmentation gene
96 boundaries, with a few exceptions. For example, between *D. melanogaster* lines with
97 variable egg size, Lott et al. (2007) found no significant differences in relative position of
98 *even-skipped* (*eve*) stripe boundaries. Even when lines with extremes of egg size were
99 crossed to generate the full range of embryo lengths between the parental lines, relative
100 segment position was invariable between different genotypes. However, between *D.*
101 *melanogaster* lines that were subjected to strong artificial selection for egg size, Miles et
102 al. (2010) found few small quantitative differences in *eve* patterning using three-
103 dimensional imaging techniques. And, Jiang et al. (2015) was able to identify a line of *D.*
104 *melanogaster* from the *Drosophila* Genetic Reference Panel (Mackay et al. 2012) with
105 altered *even-skipped* pattern formation. It is not known, however, whether these
106 changes persist beyond the embryonic stage, or if these trends appear beyond the
107 limited number of species examined. So, while segment patterning is well documented
108 to be robust, it has also been demonstrated to have some small quantitative level of

109 variation between species, and in some circumstances, within species. Evolution of this
110 robust trait does occur, and the presence of some variation within species suggests that
111 evolution of small changes may be possible without causing catastrophic failure of
112 patterning. Thus, we hypothesized that the evolution of segmentation may occur by
113 relatively small shifts, rather than rare large leaps. This would require large sample
114 sizes to detect.

115 In order to investigate the evolution of segment patterning systematically across
116 a range of divergence times, we characterized segment position across 12 species of
117 *Drosophila*. These 12 species spanned the evolutionary history of the genus (40-60
118 million years; (Russo et al. 1995; Obbard et al. 2012; Russo et al. 2013) and included
119 three pairs of sister species. We measured position of each abdominal segment relative
120 to body length, referred to throughout the manuscript as relative segment position, in
121 the first-instar larvae (Fig. 1). Larval stage is the latest developmental stage where all
122 segments are visible and easy to measure, and the use of this stage facilitated the
123 measuring of more than a hundred larvae from each species. We found that relative
124 segment positions at the larval stage have changed many times in the evolutionary
125 history of *Drosophila*, and that these changes were mostly small in magnitude, with
126 some larger changes. Most species-pair comparisons showed differences in the relative
127 position of most abdominal segments. Most sister species were significantly different at
128 every segment, however some of the most diverged species in our dataset showed no
129 differences in their patterning. The magnitude of differences in relative position
130 increased towards the posterior segments in most species, most strikingly in *D.*

131 *persimilis* and *D. mojavensis*. Phylogenetic modelling showed that rate of segment
132 position change was highly variable among the branches along the *Drosophila*
133 phylogeny, and evolutionary rate changed even between closely related species.
134 Results of a correlation analysis for position change between segments within species
135 suggested local control of segment position, as correlations decrease with physical
136 distance. Correlations of changes in the rate of evolution of segment position between
137 branches of the phylogenetic tree also decreased with physical distance between
138 segments. Overall, these results demonstrate that this complex and robust
139 developmental trait does evolve, even over short timescales, and it does so primarily by
140 small frequent steps with occasional large leaps. This may permit the evolution of novel
141 patterns over long periods of evolutionary time, without compromising the integrity of
142 this critical developmental phenotype.
143

144 Materials and Methods

145 *Species used*

146 We took relative position measurements of abdominal segments from 12 *Drosophila*
147 species spanning the evolutionary history of the genus. These species were *D.*
148 *melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. santomea*, *D. erecta*, *D.*
149 *ananassae*, *D. pseudoobscura*, *D. persimilis*, *D. willistoni*, *D. mojavensis wriglei* and *D.*
150 *virilis* (Fig. 2A). We used the sequenced lines from 11 of these species (Clark et al.
151 2007). For *D. santomea* we used stock #14021-0271.01 (The National *Drosophila*
152 Species Stock Center).

153

154 *Fly husbandry and population size control*

155 All flies were kept in plastic bottles in 20°C incubator with 60% humidity. Fly stocks were
156 raised on standard cornmeal food. The amount of time to sexual maturation varied
157 between species (Markow et al. 2008). Population control was conducted in a species-
158 specific manner through controlling the number of sexually mature adults added in a
159 given bottle, and how often the bottles were changed (Table S1). As a result, each
160 bottle ended up with 40-50 pupae. Adult flies were discarded 14 days after they became
161 fertile, to control for parental age.

162

163 *Control experiment on the effect of the larval mounting procedure*

164 In order to address whether the larval mounting procedure has species-specific effects
165 on measurements of relative segment position in first-instar *Drosophila* larvae, we
166 examined relative segment position in *D. melanogaster*, *D. sechellia* and *D. virilis* at
167 three different stages of the larval prep procedure: before heating, after heating, and
168 after mounting (Fig. S1). As these three species are variable in their egg (Markow et al.
169 2008) and larval size (Fig. 2C) and have varying phylogenetic distances (Fig. 2A), we
170 hypothesized that they might have a higher chance of being differentially affected by the
171 larval preparation protocol. First, first-instar larvae that hatched in distilled water were
172 temporarily immobilized using a 3-5 minute incubation on ice, put on a microscope slide
173 with water and then imaged using a Zeiss AxioImager microscope in brightfield. These
174 larvae were then put back in distilled water incubated at 60°C for 50 minutes and

imaged in water a second time using a brightfield microscope. The larvae were then mounted in PVA Mounting Medium (BioQuip) (see next section). The larvae were then imaged a third time using dark field microscopy (see “Imaging” section). In the images of the iced (untreated control, the icing procedure slows larvae enough for imaging) and then the heated larva, each of the 2nd and 3rd thoracic, plus eight abdominal segments were marked by a node between bulges on the larval cuticle (Fig. S1). In the dark field images, segment borders were determined by the anterior border of each denticle belt. Denticle belts are rows of bristles on the ventral side of the larvae that are used for traction while crawling (Bejsovec 2013). The anterior border of each denticle belt is a proxy for the anterior border of each abdominal segment (Lohs-Schardin et al. 1979). Using the Image J (version 1.47t) “Line tool”, measurements were taken from the anterior end of the larva to the border of each segment. Segment position was then determined by dividing this value by the full length of the larvae (Fig. 1). Segment position data was analyzed using the following linear models (using the “lm” function, all terms were fixed), implemented in R (R Core Team 2019).

Relative segment position = μ + prep + species + prep x species + ε.

Relative segment position = μ + prep + species + segment + prep x species x segment + ε.

As a result, whether we included segment as a factor (p-value = 0.07 to 0.82) or not (p-value = 0.42 to 0.83) in our linear model formula, we did not find any significant species-specific effects of the preparation protocol on segment position measurements.

197 *Preparing Drosophila first-instar larvae for imaging*

198 For each species, 20-50 newly emerged adults were obtained from a population-
199 controlled bottle and put in an egg collection bottle with a cap containing glucose-agar
200 food. For *D. sechellia,* cornmeal fly food was used in an egg lay cap with yeast sprinkled
201 on it, as this prevents this species from withholding egg laying, a particular problem with
202 this species (Markow et al. 2008). The bottle was then placed in 20°C incubator upside
203 down. The next day eggs were collected from egg lay caps, put onto a mesh and
204 thoroughly washed using distilled water to remove residual yeast and egg lay cap food.
205 The eggs were then placed in a petri dish filled with distilled water. This petri dish was
206 placed in 20°C until the eggs developed into first-instar larvae. In addition to the
207 variability in the number of days necessary to reach sexual maturity, different species
208 also varied in the number of days necessary for a fertilized egg to develop into first-
209 instar larvae (Markow and O'Grady 2005). The petri dish with water and larvae was
210 then placed in 60°C oven for ~50 minutes (Table S1), which killed and straightened the
211 larvae. These larvae were then mounted on standard glass slides using PVA Mounting
212 Medium (BioQuip), standard coverslips and a dissection microscope. Larvae were
213 oriented such that their ventral side was facing up, their posterior spiracles were
214 protruding from their body and their left-right symmetry was protected. Once the slides
215 were ready, each coverslip was sealed with clear nail polish. The slides were then
216 incubated at 60°C overnight.

217

218 *Imaging*

219 The slides were imaged at 40X objective using a Zeiss AxioImager microscope and a
220 dark field filter. Using automated tiling, 64 high resolution images were taken for each
221 larva, which were later stitched using the ZEN 2012 (blue edition) software. Each image
222 was then exported to “tagged image format”. These images will be made available prior
223 to publication.
224
225 *Image processing*
226 Measurements for the position of each abdominal denticle belt were made using a
227 custom Python script (the program will be made available prior to publication). This
228 program rotated and positioned each larva horizontally, anterior to the left and posterior
229 to the right, and cropped the image at the anterior, posterior and lateral borders. The
230 program then marked the anterior and posterior borders of the abdominal denticle belts.
231 It measured the distance from the anterior-most point of the larva to the anterior as well
232 as posterior borders of each denticle belt, and from the anterior-most point of the larva
233 to the posterior-most point of the larva (Fig. 1). Relative segment positions were
234 calculated as the distance of the anterior denticle boundary from the anterior of the
235 larva, divided by larval length (Fig. 1). The number of larval samples, from which
236 segment position measurements were taken and used for data analysis, varied from
237 105 to 145 for each species (Table S1).
238
239 *Image editing*

240 Some of the images were edited using ImageJ “Brush” tool to paint over bubbles around
241 the larvae and to adjust brightness and contrast when necessary. We found both of
242 these edits increased the number of successful runs by the custom image processing
243 program and did not change the measurements taken from these images.
244
245 *Data analysis*
246 The segment measurement data is available at
247 https://doi.org/10.6084/m9.figshare.8170787. A linear model ANOVA was fitted to the
248 data using R (R Core Team 2019) with the effects of species and segment and an
249 interaction term. “lm” and “aov” functions were used to implement the following
250 formulas. All terms were fixed.
251 Relative segment position. = μ + species + segment + species x segment + ε
252 This was followed by Tukey’s HSD (Honestly Significant Differences) (Steel et al. 1997)
253 test to conduct pairwise comparisons of the relative position of each segment between
254 species. Specifically, we used “HSD.test” function from the R package “agricolae” (De
255 Mendiburu 2019). We replicated these results using t-tests between each species pair
256 and then applying Bonferroni multiple test correction (“t.test” and “p.adjust” functions in
257 R, respectively) for both multiple species and multiple segments. These analyses were
258 done also for the posterior border and anterior-posterior width of denticle belts. For the
259 details of “posterior border” and “width” analyses of denticle belts, see File S1.
260 Principal component analysis was performed in R, using the “prcomp” function, and
261 mean centered positions of each segment as the data. To examine what PC1

262 represented in our data, we used Pearson correlation ("cor.test" function in R) to
263 correlate PC1 with the relative positions of each segment. Using the same method, we
264 also tested whether PC1 was correlated with larval length.
265 For correlation analysis between changes in relative segment position, we first
266 calculated deviation from the between-species mean of the relative position of each
267 segment in each species. We then used "cor" and "cor.test" functions in R to obtain
268 Pearson correlation coefficients and the associated p-values.
269
270 *Phylogenetic Ancestral State Estimation*
271 We performed phylogenetic analyses to infer the evolution of relative segment position
272 across the 12 *Drosophila* species here. For this, we first inferred an ultrametric
273 phylogeny published in Turelli et al. (2018) under three candidate relaxed molecular-
274 clock models. For each of the resulting phylogenies, we assessed the fit of four
275 candidate models that variously describe how rates of segment evolution vary across
276 branches of the phylogeny. Finally, we jointly inferred the phylogeny, model of segment
277 evolution, and the ancestral states for each segment under the preferred model of
278 segment evolution. We summarized various aspects of the evolution of relative segment
279 position from the resulting joint posterior probability distribution of ancestral states.
280 Complete details of these analyses are in File S2.
281

282 ## Results

283 **Larval segment allometry is highly variable across *Drosophila* species**

284 To elucidate whether relative segment position has changed over *Drosophila*
285 evolutionary history, we compared relative abdominal segment position (Fig. 1) at the
286 first-instar larval stage between 12 different species (Fig. 2A). We found that there are
287 many significant differences in relative segment position among species (Fig. 2B).
288 Overall, in 21 out of 66 species-pair comparisons, all eight segments had a significant
289 difference in their position. We note that species pairs do not represent independent
290 observations due to the underlying phylogeny (we perform a full phylogenetic analysis
291 below). For the majority of species-pair comparisons, the relative positions of five or
292 more segments differed (Fig. S2). In fact, there were only two pairs of species
293 compared (*D. simulans* - *D. ananassae* and *D. ananassae* - *D. willistoni*) where none of
294 the eight abdominal segments had a significant difference in their relative positions
295 (Table S2). Intriguingly, these species pairs are not closely related, with 15 and 32
296 million years of divergence, respectively. In all sister species comparisons, except for *D.*
297 *yakuba* – *D. santomea*, the relative position of the majority of abdominal segments were
298 different. On the other hand, between divergent species pairs, such as *D. melanogaster*
299 and *D. virilis*, relative positions of only two segments were different (Table S2).
300 Interestingly, when considering all species-pair comparisons together, some
301 species were responsible for a larger proportion of differences than others (Table S3).
302 Out of the 410 segment position differences observed over all species-pair
303 comparisons, differences with *D. persimilis* constituted 87 of these, the highest
304 proportion of any species (~21%). Differences with *D. sechellia*, *D. erecta* and *D.*
305 *mojavensis* followed with 78 each (~19%), whereas the number of differences with *D.*

306 *ananassae* was the lowest with 56 (~13.5%). This is consistent with the observation that
307 relative segment positions of *D. mojavensis* and *D. persimilis*, followed by *D. erecta* and
308 *D. sechellia*, have the highest total deviation from the species mean, whereas those of
309 *D. ananassae* are closest to the species mean making it the "average" species (Table
310 S3).

311 Next, we examined whether differences in segment position between species
312 were toward the head (anterior) or tail (posterior) of the larva. To do this, we calculated
313 mean positions of each segment across the 12 species and characterized whether a
314 particular segment in a particular species was more anterior or more posterior relative to
315 this mean (Fig. 3). For the majority of the species, the relative position of segments
316 physically closest to each other differed in the same direction. When the direction of the
317 difference changed, it was between segments that had the smallest (except for *D.*
318 *willistoni*) magnitude of difference in a given species (Fig. 3).

319 Notably, averaged over all species, deviation from the species mean in relative
320 segment position increased from anterior to posterior of the larva, with the posterior-
321 most segments having largest differences from the mean over all species. (Figs. 3,
322 S3A). Principal component analysis (Fig. S3B) also demonstrates this pattern, with PC1
323 correlating highly with the more posterior segment positions, explaining 88% of the
324 variance in the dataset. The trend of deviations in segment position increasing from
325 anterior to the posterior of the larva was strongest for *D. mojavensis* and *D. persimilis*,
326 (Figs. 3, S3C), but was nonetheless true in a number of other species as well (Fig. S3C,

327 Table S3). Indeed, when segment position data for *D. mojavensis* and *D. persimilis*
328 were excluded from the analysis, the trend still held (Fig. S3D).

329 In most species, magnitude of relative position change was highest for segment
330 A8 (Table S3), which extends from the anterior boundary of A8 to the tail of the larvae
331 (Fig. 1). We will refer to this segment as A8+tail. *D. mojavensis* and *D. persimilis* have
332 the largest differences for segment A8: *D. mojavensis* has the largest magnitude of
333 deviation from the species mean, with an anteriorly shifted segment A8 border and thus
334 a much longer A8+tail segment; while *D. persimilis* has a posteriorly shifted segment A8
335 border and thus an exceptionally short A8+tail segment. (Figs. 1, 2B and S5C). While
336 the magnitude of differences was highest for A8, the total number of differences are not
337 increased for this segment. Significant differences in relative position observed in
338 species-pair comparisons are highly similar in number for segments A3 through A8, with
339 segments A1 and A2 showing a slightly lower number of significant differences in
340 relative position (Fig. S4B, black line). Additionally, to determine if segment positioning
341 in the posterior of the embryo is less precise (noisier) as compared to the anterior, we
342 measured coefficient of variation for the position of each segment along the larva. The
343 coefficient of variation, averaged across all species, does not vary considerably and is
344 low across the length of the larva (~0.03) (Fig. S6A). This suggests that while segment
345 A8+tail has the largest magnitude differences in our dataset, its position is not more
346 variable between species, nor is it positioned any less precisely than the other
347 segments.

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

350 Given that the relative position of segment A8+tail, as compared to the other
351 segments, had the largest deviations from the species mean for most of the 12 species
352 (Fig. S3, Table S3), we asked how much of the total variation in relative segment
353 position is driven by A8+tail. In order to address this, we recalculated relative segment
354 position in the absence of A8+tail (see File S3). To determine whether any changes in
355 segment position we see was due to the removal of A8+tail region specifically, and not
356 simply due to the removal of a terminal segment, we made a separate recalculation of
357 relative segment position after removing the head and thoracic region (h+t) (Fig. 1). In
358 both cases we determined the total number of significant differences in relative segment
359 position between pairs of species (t-test with Bonferroni correction), and corrected this
360 number relative to the total number of comparisons. We found that the corrected total
361 number of significant position differences was reduced from 51.25 to 38.42 (~25%
362 decrease) when A8+tail was removed, but increased from 51.25 to 53.71 when h+t was
363 removed (Fig. S4A, compare also Fig. 2B to Fig. S7A and B). This trend held even
364 when segment position data for species with highest magnitude of posterior segment
365 position differences, *D. mojavensis* and *D. persimilis*, were removed from calculations
366 (from 31.63 down to 24, ~24% decrease, when A8+tail was removed, but up to 32.71
367 when h+t was removed). This suggests that changes in the size of A8+tail drove a
368 substantial portion of the differences in relative segment position between species. On
369 the other hand, changes in the size of h+t appear to have masked some of the inter-

370 species differences in relative position observed in the rest of the segments. These
371 results are consistent with the finding that the average difference in relative position of
372 A1, and hence the difference in the size of h+t, are the smallest in magnitude among all
373 segments (Figs. S3A, S5B), while relative position of A8, hence the size of A8+tail, has
374 the largest magnitude changes (Figs. S3A, S5C). They are also consistent with the
375 finding that total number of significant position changes are lower for segment A1 than
376 they are for A8 (Fig. S4B). For a more detailed analysis of "end removal" and its effects
377 as well as differences in the size of h+t and A8+tail, see File S3.
378

379 **Changes in the length of larval body**

380 As we had collected larval length measurements in order to calculate relative
381 segment positions, we also compared whole body length at the first-instar larval stage
382 among 12 *Drosophila* species (Fig. 2C), and tested whether length had an effect on
383 relative segment positioning. *D. santomea* was the largest at this stage, followed by *D.*
384 *sechellia* and *D. virilis*. *D. persimilis* and *D. pseudoobscura* were the smallest, followed
385 by *D. mojavensis*. Body length for the rest of the species showed few and smaller
386 differences (Fig. 2C). We had two comparisons between sister species with large size
387 differences, as *D. santomea* and *D. sechellia* were among the largest larvae, while their
388 sisters, *D. yakuba* and *D. simulans*, respectively, were of roughly average size. Sister
389 species *D. santomea* and *D. yakuba* had few differences in segment position, whereas
390 sister species *D. sechellia* and *D. simulans* were different for every segment, suggesting
391 that larval size is not predictive of segment position differences. Overall, larval length is

392 not correlated with number of segment position differences (Fig. S5A, R^2 = 0.0016, p-
393 value = 0.75). In other words, having a bigger difference in body length was not
394 correlated with having more differences in relative segment position between species.
395 Additionally, larval length had no effect on the direction (anterior vs. posterior) of
396 segment position differences, as we detected no relationship between body length and
397 the number of segments that are shifted to the anterior or posterior compared to the
398 species mean (Wilcoxon test, p-value = 1). Finally, we returned to our PCA (above), and
399 found that PC1, which was highly correlated with the more posterior segment positions,
400 was uncorrelated with larval length (Pearson correlation, p-value = 0.06).
401
402 **Phylogenetic analysis shows variable rates of segment position evolution**
403 To investigate how relative segment position has evolved during *Drosophila*
404 evolutionary history, we implemented phylogenetic methods to estimate the rates of
405 morphological evolution over the phylogeny. We employed a multivariate Brownian
406 motion model of evolution (Huelsenbeck and Rannala 2003; Lartillot and Poujol 2010),
407 and tested various morphological branch rate prior models that describe how rates of
408 morphological evolution vary across the branches of the tree. These analyses were
409 performed in RevBayes (v. 1.0.7; Höhna et al. 2016), and are outlined in detail in File
410 S2. Of the candidate models of segment evolution that we explored, the data
411 significantly preferred the uncorrelated lognormal relaxed molecular clock model, which
412 allowed rates to vary episodically along ancestor-descendant branches (*i.e.*, rates of
413 segment evolution are not correlated between ancestor-descendant branches). Results

414 presented here are for this model, but other morphological evolutionary models gave
415 similar results (File S2).

416 Our phylogenetic analyses indicate that evolutionary rates of relative segment
417 position are highly variable across branches of the *Drosophila* phylogeny (Figs. 4A,
418 S8A and B). Many of the highest rates of evolution are on branches for species with
419 sister species included in the analysis (i.e. *D. sechellia* and *D. simulans*; *D. santomea*
420 and *D. yakuba*; *D. persimilis* and *D. pseudoobscura*). This points to segment position
421 evolving quickly between these sibling species. The patterns vary among segments
422 (Fig. S9). Two lineages that are also evolving rapidly, particularly in the posterior half of
423 the larva (Figs. 4A, S9), those leading to *D. mojavensis* and *D. persimilis*, have the
424 longest and shortest posterior-most segment (A8+tail), respectively, in our dataset
425 (Figs. 2B, S5C). It is intriguing that the branch leading to *D. mojavensis* showed one of
426 the fastest rates of evolution, as it is also on a long branch. This high rate sustained
427 over a long branch is consistent with the A8+tail segment of *D. mojavensis* having the
428 largest magnitude change in our dataset (Figs. 2B, S3C).

429 Given that several species had large changes in segment position toward the
430 posterior of the larvae (Figs. 2B, 3, and S3C), we asked whether the overall rate of
431 evolution is also higher for some segments than it is for others. We examined the rate of
432 position evolution for each segment over all branches of the tree and found that rate of
433 evolution varies among segments, with posterior segments exhibiting elevated rates
434 (Fig. 4B). Segment A8 had the highest rate of change in segment position, with
435 segment A7 also having a slightly elevated rate than the rest of the segments (Fig. 4B).

436 The high rate of evolution for A8 is consistent with some of the largest magnitude
437 changes in our raw data (Figs. 2B, 3) being found for this segment.

438 To determine whether relative segment position has evolved through frequent
439 small changes or rare large changes, we examined the distribution of the amount of
440 change in relative segment position normalized across all branches of the *Drosophila*
441 evolutionary tree (i.e. amount of change per unit time; see File S2, S.3.4.5 and S.3.4.6
442 for details). This distribution of normalized magnitude of change in segment position
443 across all the branches of the phylogeny (Fig. 4C) showed that most segment positions
444 on most branches have experienced changes that are small in magnitude, whereas
445 some segment positions on some branches have experienced substantially larger
446 changes within a given amount of evolutionary time. This analysis shows that
447 throughout *Drosophila* evolutionary history, relative segment position has changed
448 predominantly through small quantitative steps, with occasional large leaps.

449

450 **Is segment position along the anterior posterior axis controlled locally or**
451 **globally?**

452 Within each species, we asked how the shift in the relative position of one
453 segment might be correlated to shifts in the relative position of other segments. This
454 would indicate whether there is a focal point along the anterior-posterior axis controlling
455 segment position or whether there is a more complex underlying regulatory mechanism.
456 We found that within all 12 species, the correlation between shifts in the relative
457 positions of any two segments is inversely proportional to the distance between the two

458 segments (Figs. 5A, S10, and S11). We did not observe a focal point governing the shift
459 in segments, where it might be expected that correlations would drop off as the distance
460 from that particular point increased. It appears that small adjustments in segment
461 position are made locally. These correlations are looking at the relationship between
462 segment positions across individuals within a species. As the variation within species
463 reflects developmental variation in a genetically identical line, these correlations show
464 that developmental variation in the positioning of one segment have stronger effects on
465 nearby segments than more distant segments. This is consistent with evidence from the
466 embryonic stage that showed correlation between deviation from mean in expression
467 boundary for various segmentation genes decreased with increasing distance between
468 the expression boundary (Lott et al. 2007). Interestingly, averaged over all species, shift
469 in the relative position of A8 was less well-correlated to shifts in the rest of the segments
470 (Figs. 5B, S12). This suggests that shifts in the relative position of A8 are more
471 independent than the shifts in the relative position of other segments within species
472 (See also File S4).

473 Given that the relative position of A8+tail changed more independently within a
474 species as compared to the other segments (Fig. 5B), we asked whether relative
475 position would be more tightly regulated across the other segments in the absence of
476 A8+tail or whether A8+tail was essential for proper segment positioning. To address this
477 question, we calculated correlation coefficients using relative segment positions
478 determined in the absence of A8+tail. These recalculated correlations resulted in lower
479 average correlation coefficients between position shifts of adjacent (as well as more

480 distant) segments as compared to when all segments were included in position
481 calculations. (Figs. S13, S14, red line, *D. sechellia* was exceptional, see Fig. S15). This
482 was also true when we recalculated correlations using relative segment positions
483 determined in the absence of h+t (Figs. S13, S14, blue line). These results suggest that
484 both ends of the larvae are needed for proper segment positioning, and removal of
485 either one decreases the level of coordination between segments, as demonstrated by
486 the reduction in correlation coefficients.
487
488 **Coordination of segment position evolution across the larva**
489 Within a species, segment position seems to be controlled locally in response to
490 developmental variation, as correlations between segments decrease with physical
491 distance in the larva. How then are evolutionary segment position changes correlated
492 across species? To address this question, we used the phylogenetic model to estimate
493 correlations in the rate of position change between segments across species. These
494 phylogenetic correlations may reflect underlying genetic correlations (in a quantitative
495 genetic sense) or be the result of constraints placed on the system by selection
496 pressures. Evolutionary rates of relative segment position were highly correlated across
497 the branches of the phylogeny, and correlation decreased as the anatomical distance
498 between segments increased (Fig. 5C). All correlations were positive, indicating that, for
499 example, when rapid evolution occurs in one segment, changes occur across all
500 segments. However, across species, the rate of evolution of segment A8 is not less
501 correlated than expected, given its distance from other segments. This suggests that

502 while this segment may evolve at a faster rate (Fig. 4B), on average it does not seem to
503 be evolving in a less coordinated fashion than the other segments, when the whole
504 phylogeny is considered.
505

506 ## Discussion

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

513 Given the suppression of phenotypic variation in robust traits, their evolution has
514 been a subject of considerable interest to researchers over the years, and has produced
515 both theoretical and empirical work (Wagner 2005; Félix and Wagner 2006; Masel and
516 Siegal 2009; Siegal and Leu 2014; Payne and Wagner 2019). One proposal for the way
517 for a robust trait to evolve is for conditions to exceed the tolerance for variation in that
518 trait, and expose genetic variation that had previously been masked, i.e. cryptic genetic
519 variation, by the very robustness of the trait (Rutherford and Lindquist 1998; Gibson and
520 Dworkin 2004; Paaby and Rockman 2014). Exposing any amount of genetic variation of
521 unknown consequence to a critical trait at a time of great stress seems like a dangerous
522 proposition in a multicellular animal. Our data supports an alternative model, where

523 there is always some small amount of variation available, even in the most robust traits,
524 and that quantitative changes may evolve in robust traits without disrupting robustness.
525 Here, we focus on a well-studied robust process and trait, segmentation in
526 *Drosophila*. Previous studies have highlighted the ability of the segmentation network to
527 produce precisely localized segment markers in embryogenesis despite experimentally
528 produced perturbations in development (Félix and Wagner 2006; Masel and Siegal
529 2009). Our study extends this to measure the trait of segmentation in larvae, across 12
530 *Drosophila* species. Here, we demonstrate that segmentation varies considerably
531 across species, and that the rate of evolution of segment position varies considerably
532 across the phylogeny as well. The variation in segment position consists mostly of small
533 magnitude differences, suggesting that this trait evolves by many small changes, with
534 occasional larger changes. While it can be difficult to quantitatively compare earlier
535 stages of segmentation in the embryo to segments in the larva, this result is consistent
536 with patterning differences in the embryo observed between species (Lott et al. 2007;
537 2010). Our data shows that segment position differences between species exist also in
538 later developmental stages. Moreover, each line of each species measured here
539 precisely produces its characteristic segmentation pattern in the larva. This points to the
540 ability of segmentation to evolve without losing its ability to be precisely localized (Lott et
541 al. 2010). While we did not test the robustness of each species to genetic and
542 environmental variation specifically, this also suggests that segmentation can evolve
543 and remain robust to stochastic variation. If segmentation can evolve small quantitative
544 changes over evolutionary time without losing robustness, then this suggests that not all

545 genetic variation in this trait is cryptic (Miles et al. 2010; Jiang et al. 2015), and small-
546 scale differences (and the occasional larger-scale difference) in segmentation patterns
547 may be available to selection (Weber 1992). Or, perhaps there is a neutral space where
548 a range of segment positions are tolerated, and stabilizing selection keeps them in that
549 range.

550 The evolutionary patterns observed here may also be simply what might be
551 expected of complex traits generally, with changes in a number of genes producing
552 small quantitative changes in phenotype over evolutionary time. Here, we find that
553 segment position seems locally determined along the length of an individual, as position
554 shifts are highly correlated between adjacent segments, and correlations drop off over
555 distance, consistent with a previous result within species in embryos (Lott et al. 2007).
556 This is also consistent with the known regulatory interactions in this network, where
557 patterns are refined over developmental time (DiNardo and O'Farrell 1987; Surkova et
558 al. 2008) and regulation of segment patterning becomes more localized to a smaller
559 portion of the developing animal at each stage as compared to the previous one
560 (Nüsslein-Volhard and Wieschaus 1980; Kornberg and Tabata 1993; Nüsslein-Volhard
561 et al. 2008). Our results suggests that changes in many genes produced the observed
562 differences observed between species, and hence, it may be difficult to determine the
563 identity of individual genetic changes that may cause the differences in segment
564 position. However, the genetic network behind segment patterning has been fertile
565 ground for modeling in the embryo (Jaeger et al. 2004; Manu et al. 2009a; Wunderlich
566 and Depace 2011; Bentovim et al. 2017; Clark 2017; Verd et al. 2018; Petkova et al.

567 2019). Implementing a modeling framework developed for the embryonic stage, and
568 extending it past the embryonic segment polarity stage to the positions of segments in
569 the larva, is likely the best approach for identifying potential genetic causes of the
570 differences observed here.

571 Across the larva, the most striking pattern we observed was in the posterior-most
572 segment, from A8 to the tail of the animal. We found that this segment has the largest
573 magnitude of differences between species, and evolves faster between species than the
574 other segments. It is the most independently controlled segment within a species, with
575 correlations between its position and all other segments being the lowest found. As the
576 h+t region anterior of A1 does not share these properties, it is not simply a feature of
577 terminal segments, but specific to the A8+tail region. The A8+tail region contains the
578 posterior spiracles, important breathing structures in the larva (Hu and Castelli-Gair
579 1999), as well as the genital imaginal disc from which the genital structures in the adult
580 are produced (Sánchez and Guerrero 2001). While genital structures are known to
581 evolve rapidly between species (Eberhard 2013), it is unclear whether this would
582 produce differences in the entire segment in which the genital imaginal disc is found.
583 Alternatively, the differing conditions in which larvae find themselves may produce
584 differences in behavior that would require differences in posterior spiracle length. We
585 are currently exploring this possibility in relatives of *D. mojavensis*, as this species has
586 an exceptionally long A8+tail segment. In our care, *D. mojavensis* larvae burrow deeply
587 in food with only their long posterior spiracles visible above the surface of the food. As
588 *D. mojavensis* and its relatives are desert-dwelling cactus specialists (Oliveira et al.

589 2012), perhaps the larvae burrow deeply into cactus in their natural environment to find
590 a more hospitable microclimate (McKenzie and McKechnie 1979; Green et al. 1983).

591 Overall, our results show for the first time that segment position in *Drosophila* has
592 changed frequently throughout the evolutionary history of the genus. The changes were
593 mostly small in magnitude, presumably representing only small perturbations to the
594 development of the organism. Rate of evolution of this trait varies across the phylogeny,
595 with larger magnitude differences and a higher rate of evolution observed for the tail of
596 the larva. Changes in segment position within species, as well as rates of evolution
597 between species, are highly correlated between neighboring segments, indicating the
598 highly coordinated nature of the genetic network underlying this trait. Future studies are
599 needed to unravel the nature of the genetic changes underlying segment position
600 differences between species and whether any of the observed phenotypic differences
601 between species might be adaptive.

602

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604

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851
852
853 **Figure 1.** Example larval images and description of measurements. Example dark field
854 images of first instar larvae are shown for three species. *D. ananassae* is presented as
855 it is closest to the mean of all species for segment positions, while *D. persimilis* and *D.*
856 *mojavensis* are exceptional, especially in the length of their most posterior segments.
857 Red lines mark the anterior border and white lines mark the posterior border of denticle
858 belts, which are rows of bristles on the ventral surface of the animal and are recognized
859 by our image processing program. The anterior border of the denticle belt was used as
860 a proxy for segment border. To measure the position of an abdominal segment (e.g. A1)
861 relative to the body length (Y), the distance from the anterior border of the larvae to the
862 anterior border of that segment (X) is divided by the total body size (Y). Region
863 encompassing Head, T1, T2, T3 is referred to as “h+t” throughout the main text. T =
864 Thoracic Segment, A = Abdominal Segment.
865
866 **Figure 2.** Relative segment position and body size is highly variable among 12 species
867 of *Drosophila*. (A) 12 *Drosophila* species on an evolutionary tree. Adult male species
868 photos from Nicolas Gompel were downloaded from FlyBase. (B) Each black bar
869 represents the mean position in percent larval length for each segment in each species.
870 The color spread on the two sides of each black bar is 95% confidence interval. Each
871 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. Coordinated direction of relative segment position changes in 12 *Drosophila* species. Neighboring segments tend to shift together in a particular direction, toward the anterior or posterior of the larva rather than shifting in opposite directions. Yellow indicates anterior, blue indicates posterior shift in relative position as compared to the mean position across all species for a given segment. The area of the circle is proportional to the size of the shift in relative segment position.

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. Rates are variable across the phylogeny, with some big differences in rate observed even between closely related species. (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. The rate is higher for the most posterior segments, especially A7 and A8 (C) Marginal posterior probability distribution of the evolutionary rates of relative segment position across branches. Most changes are small in magnitude, with occasional larger changes.

Figure 5. Correlations between segment positions, within and between species. (A) Within a species (here, *D. melanogaster* is shown as an example), correlations between relative segment positions are highest for neighboring segments, and fall off as the physical distance between two segments increases. Colors are used to distinguish between segment pairs with different number of segments separating them. This suggests local control of segment positioning. For plots for the rest of the species see Fig. S10. (B) This plot is similar to that in part A, but instead shows averages of correlation coefficients between segment positions over all species. Similarly, correlations between relative segment positions are highest between neighboring segments, but fall as distance between segments increase. In red are all comparisons with segment A8. At any distance, correlations with this segment are lower (in black are all other comparisons not involving A8). The gray and red shaded areas around the black and red points, respectively, indicate their 95% confidence intervals. This demonstrates that changes in the position of A8 are less correlated, and thus are more independent, than changes in the position of the rest of the segments, given its distance from any other segment. The unit of the x-axes in panels A and B, for mean relative distance between segments, is percent larval length. (C) Between species, correlations between evolutionary rates for a pair of segments are lower with increasing distance between segment pairs. This plot represents a similar analysis to part B, but in the phylogenetic framework and correlates rates of evolution for each segment. 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. Rates of evolution are highly correlated between neighboring segments, and correlations decrease with increasing distance between segments.

Figure S1. Testing the effect of larval mounting procedures on relative segment position. For this experiment, relative segment position measurements were taken before and after multiple stages of the mounting protocol, to test the effect of the mounting protocol on segment position across multiple species (see Methods) (A) Bright field image of a *D. melanogaster* first instar larva, hatched in water, iced for 2-3 minutes before imaging. (B) The same *D. melanogaster* larva heated at 60°C for an hour prior to bright field imaging. (C) Dark field image of the same *D. melanogaster* larva, mounted using PVA, and incubated in the 60°C oven overnight. This represents the end stage of our mounting protocol. White horizontal lines mark the full length of the larvae, vertical lines mark the segment borders.

Figure S2. Comparing pairs of species, most segments were in different relative positions. For the majority of species pair comparisons, five or more segments had significantly shifted relative positions (t-tests with Bonferroni correction, p-value ≤ 0.05). The graph shows the number of species pairs on the y-axis and number of segments significantly shifted (see Methods) on the x-axis.

Figure S3. The posterior-most segments show the largest magnitude in differences from mean across species. (A) Average absolute magnitude of shift in the relative position of each segment, as compared to the across species mean, 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) From principal component analysis, PC1 is plotted for all species. Positive values correspond to posterior shifts of segments, negative values indicate anterior shifts of segments, relative to mean centered positions for each segment. PC1 explains 88% of the variance and is highly positively correlated with the positions of the most posterior segments (Pearson correlation coefficients with each segment, A1-A8: A1= -0.15, A2=0.11, A3=0.28, A4=0.49, A5=0.64, A6=0.77, A7=0.85, A8=0.88). Note the similarity in pattern to Figure 3, with *D. mojavensis* having the largest deviation from the rest of the species, with a dramatic shift toward the anterior (negative PC1 values) and *D. persimilis* shifted toward the posterior (positive PC1 values). (C) 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 in the graph shows the slopes calculated for each trend form anterior to posterior. Lines in the graph and the legend are color coordinated for each species. (D) Average absolute magnitude of shift in the relative position of each segment, as compared to the across species mean, increases from anterior to the posterior of the larvae even in the absence of segment position data for *D. persimilis* and *D. mojavensis* (compare to Figure S3A, this representation is the

same, except with *D. persimilis* and *D. mojavensis* removed from the calculation of the mean). 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.

Figure S4. The A8+tail region is responsible for much of the number and magnitude of significant differences between species. For all these plots, compare the effect of when A8+tail is removed to when another terminal segment (h+t) is removed, to determine whether the effect is A8+tail specific or simply an effect of removing a terminal segment. (A) Comparison of numbers 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) This plot shows the same data as part A, but illustrates how the differences in number change across the length of the larva. 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 number of significant changes in relative position normalized to the number of segments included in position calculations. (C) Average absolute 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.

Figure S5. Features of body length. (A) Average body length difference between a given pair of species is not correlated with the number of segments with significant position shifts between the species. The 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 h+t, 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. Parts B and C represent transformations of the position and length measurements, to highlight the length of the terminal portions of the larva.

1004
1005 **Figure S6.** Coefficient of variation in segment positioning stays low throughout the
1006 larva, indicating that the relative positions of all segments are equally as precisely
1007 determined. However, standard deviation of segment positioning increases towards the
1008 posterior end of the larva, as the measurement values increase (relative position is
1009 measured in % of larval length from the anterior end). (A) Change in coefficient of
1010 variation, averaged over 12 *Drosophila* species, for all segments from anterior to
1011 posterior of the larva. The y-axis shows coefficient of variation, and the x-axis depicts
1012 each abdominal segment. When segment positions were calculated with all segments
1013 included (black dots), coefficient of variation (noise) did not change along the anterior-
1014 posterior axis of the larva, and remained low. Removal of A8+tail (red dots) overall
1015 decreased noise levels in segment positioning, whereas removal of h+t (blue dots)
1016 increased noise levels towards the posterior of the larva. (B) Change in standard
1017 deviation, averaged over 12 species, for all segments from anterior to posterior of the
1018 larva. The y-axis shows standard deviation and the x-axis depicts each abdominal
1019 segment. When segment positions were calculated with all segments included (black
1020 dots), standard deviation of segment positions increased towards the posterior of the
1021 larva. Removal of A8+tail (red dots) decreased standard deviations in the posterior,
1022 whereas removal of h+t (blue dots) increased standard deviations towards the posterior
1023 of the larva.
1024
1025 **Figure S7.** Relative segment position and body size is highly variable among 12
1026 species of *Drosophila,* though less so when A8+ tail is removed. These graphs have the
1027 same format as Figure 2B and shows differences in relative segment position between
1028 species. (A) Relative segment positions are calculated in the absence of A8+tail. Notice
1029 a substantial number of nonsignificant differences as compared to Figure 2B. (B)
1030 Relative segment positions are calculated in the absence of h+t and also with
1031 measurements taken from the posterior of the larva instead of the anterior. As
1032 compared to Figure 2B the segment order is flipped, but the pattern of change in
1033 segment position is very similar. In both graphs, each black bar depicts the mean for
1034 each segment in each species. The colored areas are 95% confidence intervals. The y-
1035 axis depicts 12 *Drosophila* species and x-axis shows mean relative abdominal segment
1036 position in percent larval length.
1037
1038 **Figure S8.** Phylogenetic analysis of relative segment evolution in the 12 *Drosophila*
1039 species, with posterior rate estimates for all branches. A) Like Figure 4A, the phylogeny
1040 was inferred from nuclear loci with relative divergence times, and branches of the tree
1041 are colored to indicate the overall rate of relative segment position evolution. Here, the
1042 branches are numbered to correspond with the values pictured in part B. B) Box plot
1043 depicting rate of evolution estimate for each branch of the phylogeny, x-axis
1044 corresponds to the branch numbers indicated on the tree in part A.
1045
1046 **Figure S9.** Phylogenetic analysis of relative segment position for each segment. Like
1047 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 Figure S8, 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.

Figure S10. Correlations between relative segment positions within each species. This series of graphs are constructed the same as Figure 5A, which was for *D. melanogaste*r, for each of the 12 *Drosophila* species. The y-axis shows correlation coefficients and the x-axis shows mean relative distance between pairs of segments in percent larval length.

Figure S11. Correlation coefficient heat maps for each of the 12 *Drosophila* species. This shows the correlation coefficient between shifts in the relative position (as compared to the across species mean) of each pair of segments within a species. Red indicates high positive correlation and white indicates low positive correlation. Pearson correlation coefficients 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 a lower correlation with shifts in the position of other segments, regardless of the distance in between any pair of segments.

Figure S12. Correlations between segment positions, comparing correlations including segment A8 with all other pairwise comparisons the same number of segments apart. This series of graphs represent the information from Figure 5B, but broken out by how many segments separate the two segments being compared. Each graph has mean correlation coefficient over all species on the y-axis, and mean relative distance in percent larval length over all species on the x-axis. Segments that are paired with A8 are in red. Error bars are 95% confidence intervals.

Figure S13. When all segments are included in position calculations, mean correlation coefficients between neighboring segments 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.

Figure S14. This series of graphs are a continuation of Figure S13, as the distance between pairs of segments increase, from two to six segments apart. Notice that removal of h+t overall decreases correlation coefficients more than removal of A8+tail.

Figure S15. This series of graphs show the data represented in Figure S13 separately for each of the 12 *Drosophila* species. They show, for each species, how correlation coefficients 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).

Table S1. Experimental methods used for 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.

Table S2. Lists the number of segments that are differentially positioned between pairs of species, and the divergence time between each pair in millions of years (Russo et al. 1995; Obbard et al. 2012; Russo et al. 2013).

Table S3. Lists the deviation of the position of each segment in each species from the "across-species" mean as well as total number of significant segment position changes for each species over all species-pair comparisons.

File S1. Describes the observed changes in the position of posterior border of denticle belts and denticle width between species.

File S2. Methods and results of the phylogenetic analysis.

File S3. Description of the method for removal of A8+tail or h+t from segment position calculations, as well as some additional results from that analysis. In particular, this file highlights the effects of the new calculations on differences in segment position along the larva as well as the variation in segment position within a species. It also gives more detail on the differences in the size of A8+tail and h+t between species when all segments are included in segment position calculations.

File S4. Describes correlation between changes in the position of adjacent segments along the anterior-posterior axis for each species, highlighting species-specific patterns.

Figure 1

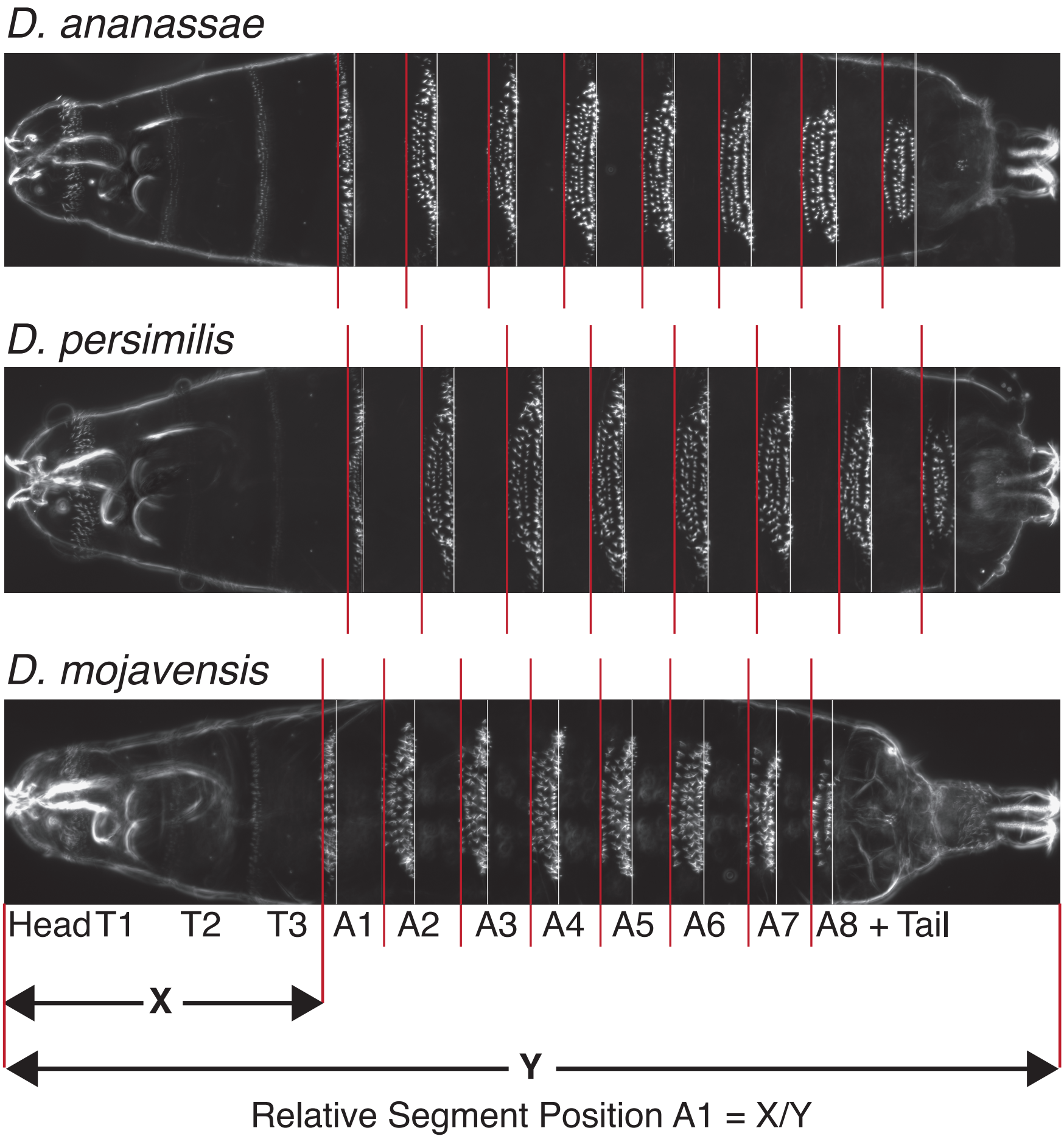
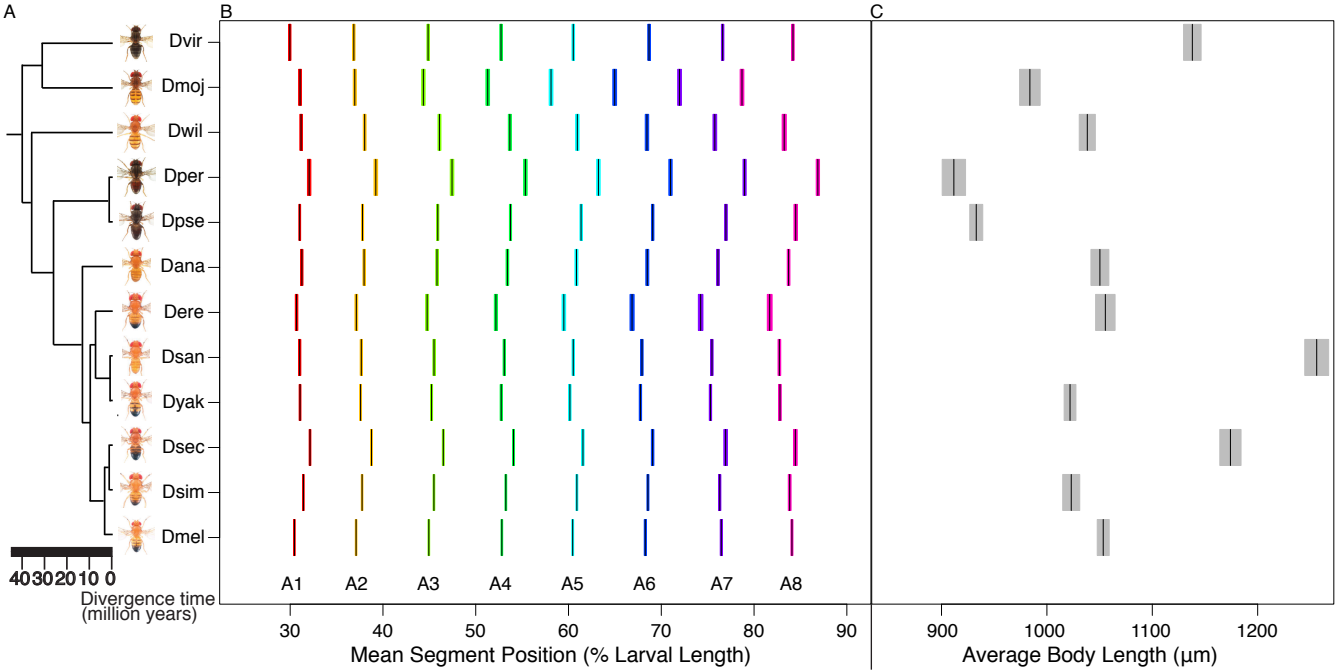

D. ananassae
D. persimilis
D. mojavensis
Head
T1
T2
T3
A1
A2
A3
A4
A5
A6
A7
A8 + Tail
X
Y
Relative Segment Position A1 = X/Y

Figure 2


A
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C
Dvir
Dmoj
Dwil
Dper
Dpse
Dana
Dere
Dsan
Dyak
Dsec
Dsim
Dmel
40 30 20 10 0
Divergence time
(million years)
A1
A2
A3
A4
A5
A6
A7
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Mean Segment Position (% Larval Length)
900
1000
1100
1200
Average Body Length (μm)

Figure 3

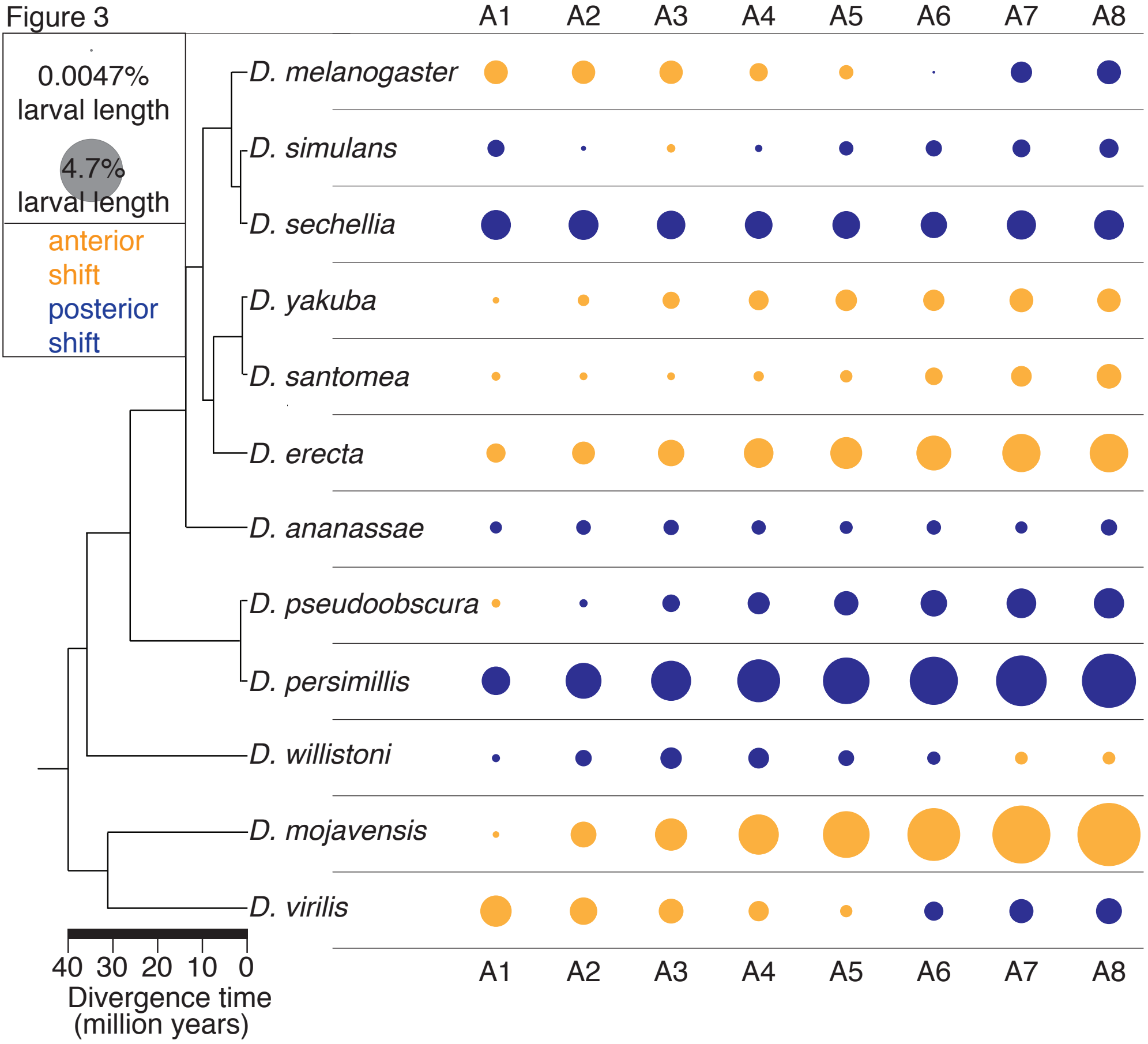
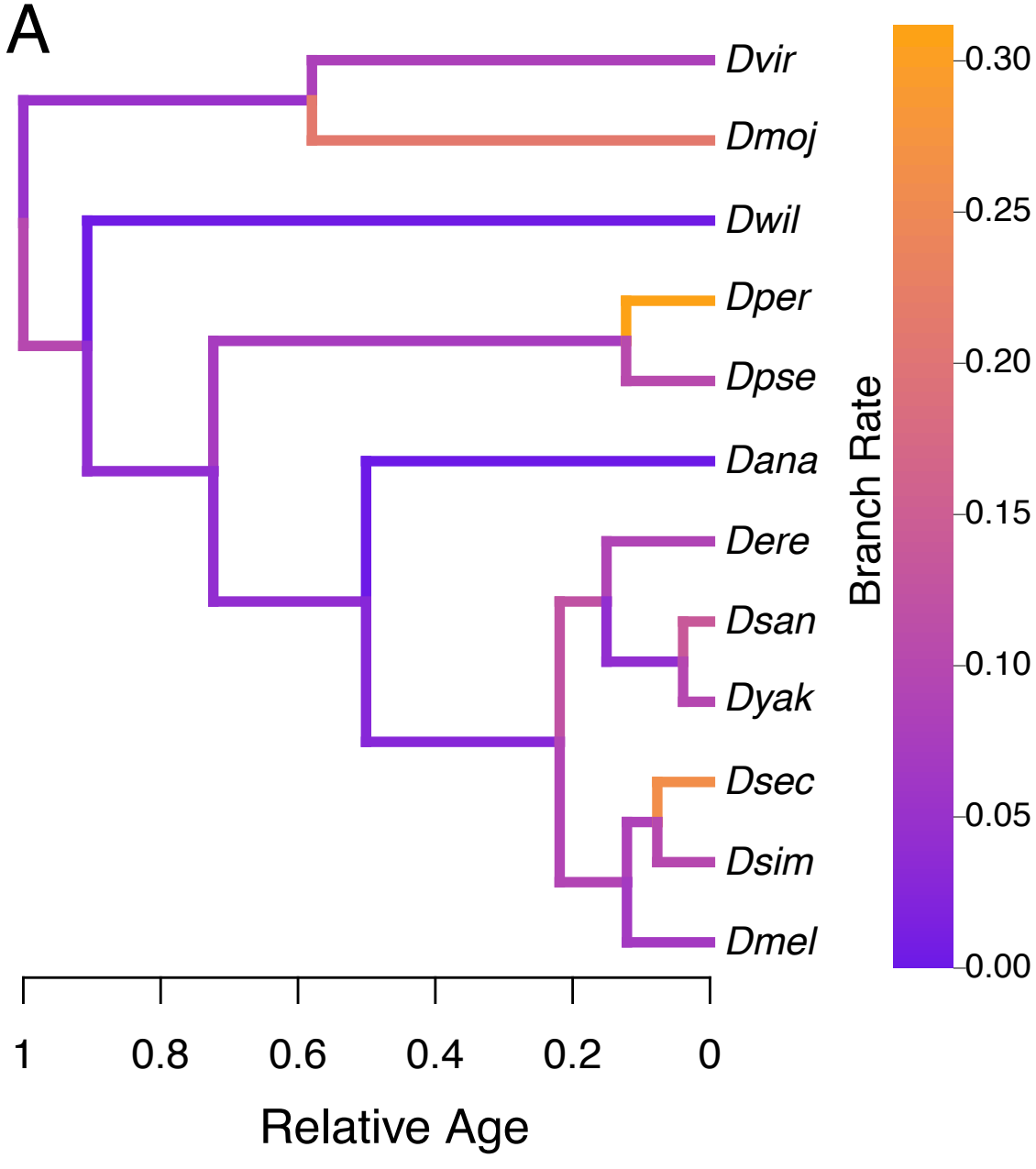
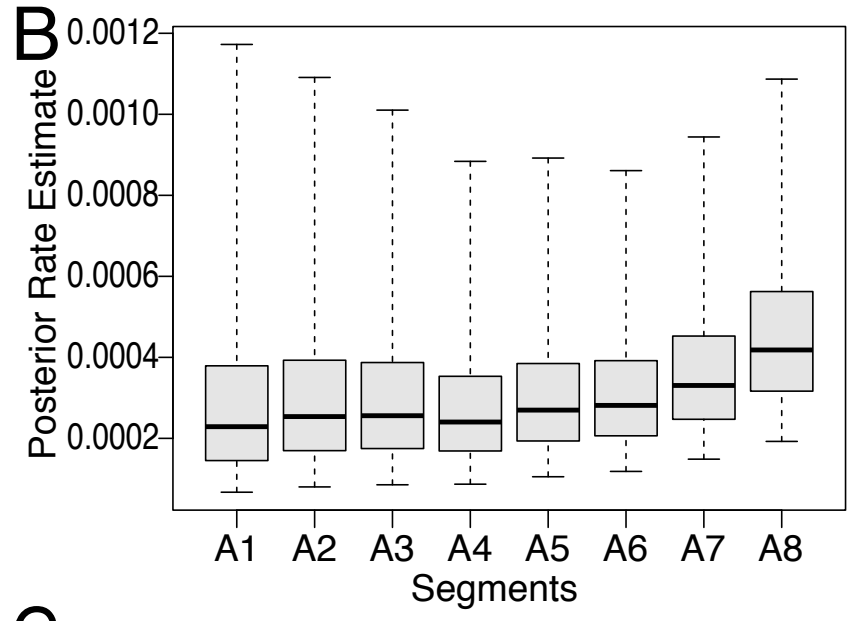

A1
A2
A3
A4
A5
A6
A7
A8
0.0047%
larval length
4.7%
larval length
anterior
shift
posterior
shift
D. melanogaster
D. simulans
D. sechellia
D. yakuba
D. santomea
D. erecta
D. ananassae
D. pseudoobscura
D. persimillis
D. willistoni
D. mojavensis
D. virilis
40 30 20 10 0
Divergence time
(million years)

Figure 4


A
Dvir
Dmoj
Dwil
Dper
Dpse
Dana
Dere
Dsan
Dyak
Dsec
Dsim
Dmel
1
0.8
0.6
0.4
0.2
0
Relative Age
Branch Rate
0.30
0.25
0.20
0.15
0.10
0.05
0.00


B
Posterior Rate Estimate
0.0012
0.0010
0.0008
0.0006
0.0004
0.0002
A1
A2
A3
A4
A5
A6
A7
A8
Segments

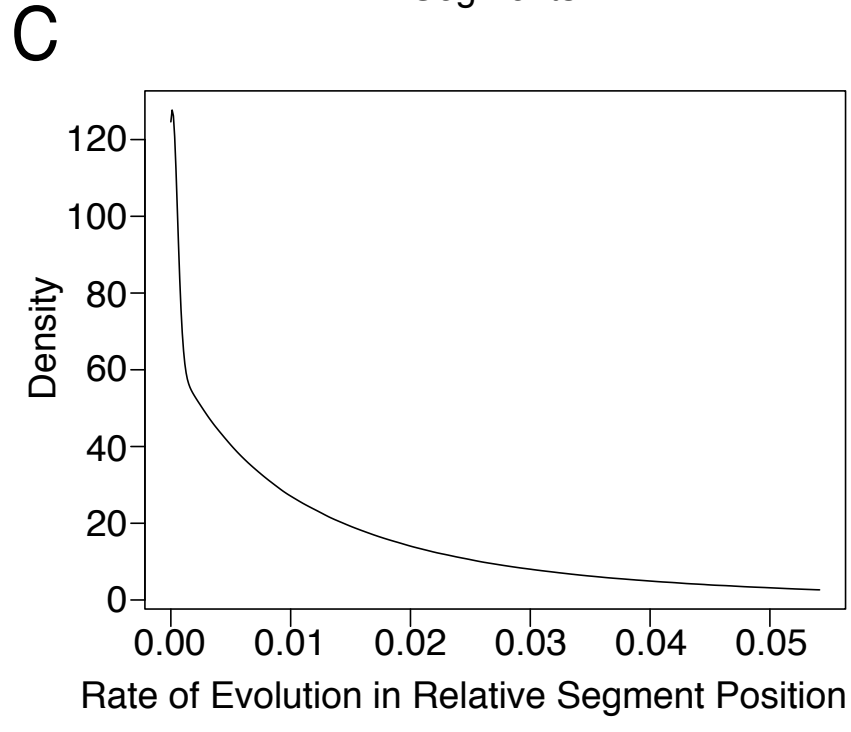
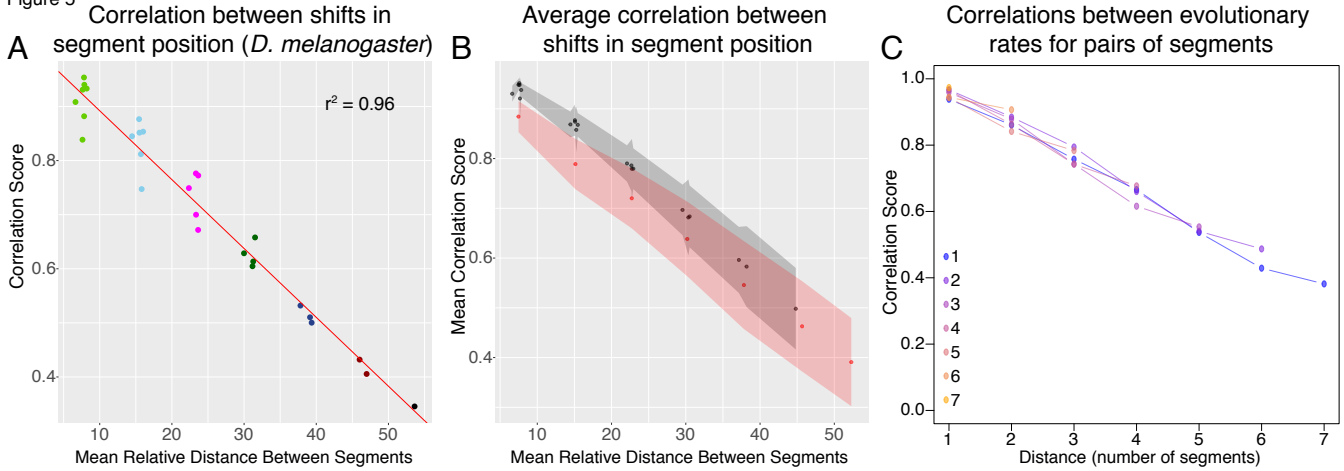
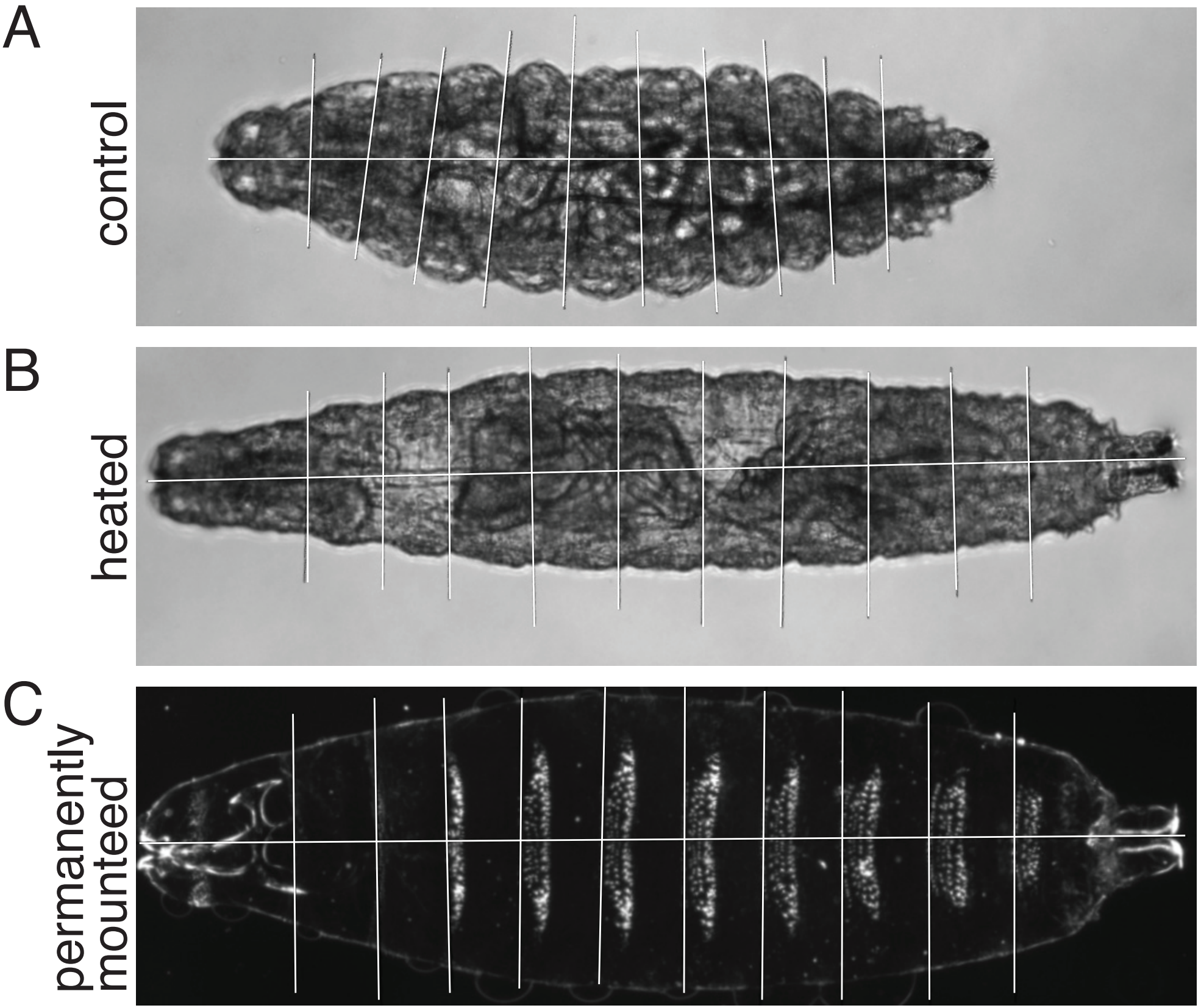

C
Density
120
100
80
60
40
20
0
0.00
0.01
0.02
0.03
0.04
0.05
Rate of Evolution in Relative Segment Position

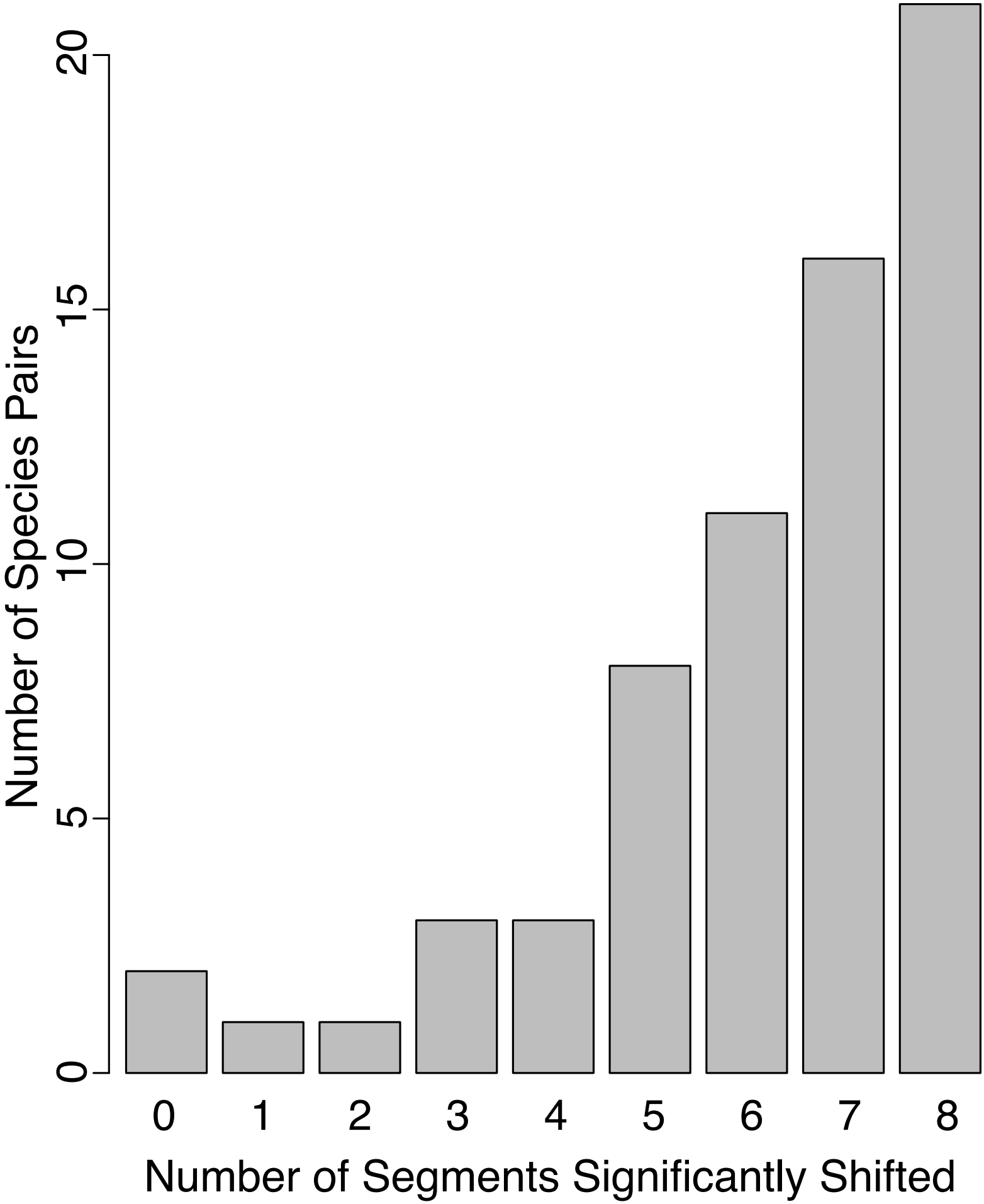
Figure 5


A
Correlation between shifts in segment position (D. melanogaster)
r² = 0.96
Correlation Score
0.8
0.6
0.4
10
20
30
40
50
Mean Relative Distance Between Segments
B
Average correlation between shifts in segment position
Mean Correlation Score
0.8
0.6
0.4
10
20
30
40
50
Mean Relative Distance Between Segments
C
Correlations between evolutionary rates for pairs of segments
Correlation Score
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
6
7
Distance (number of segments)

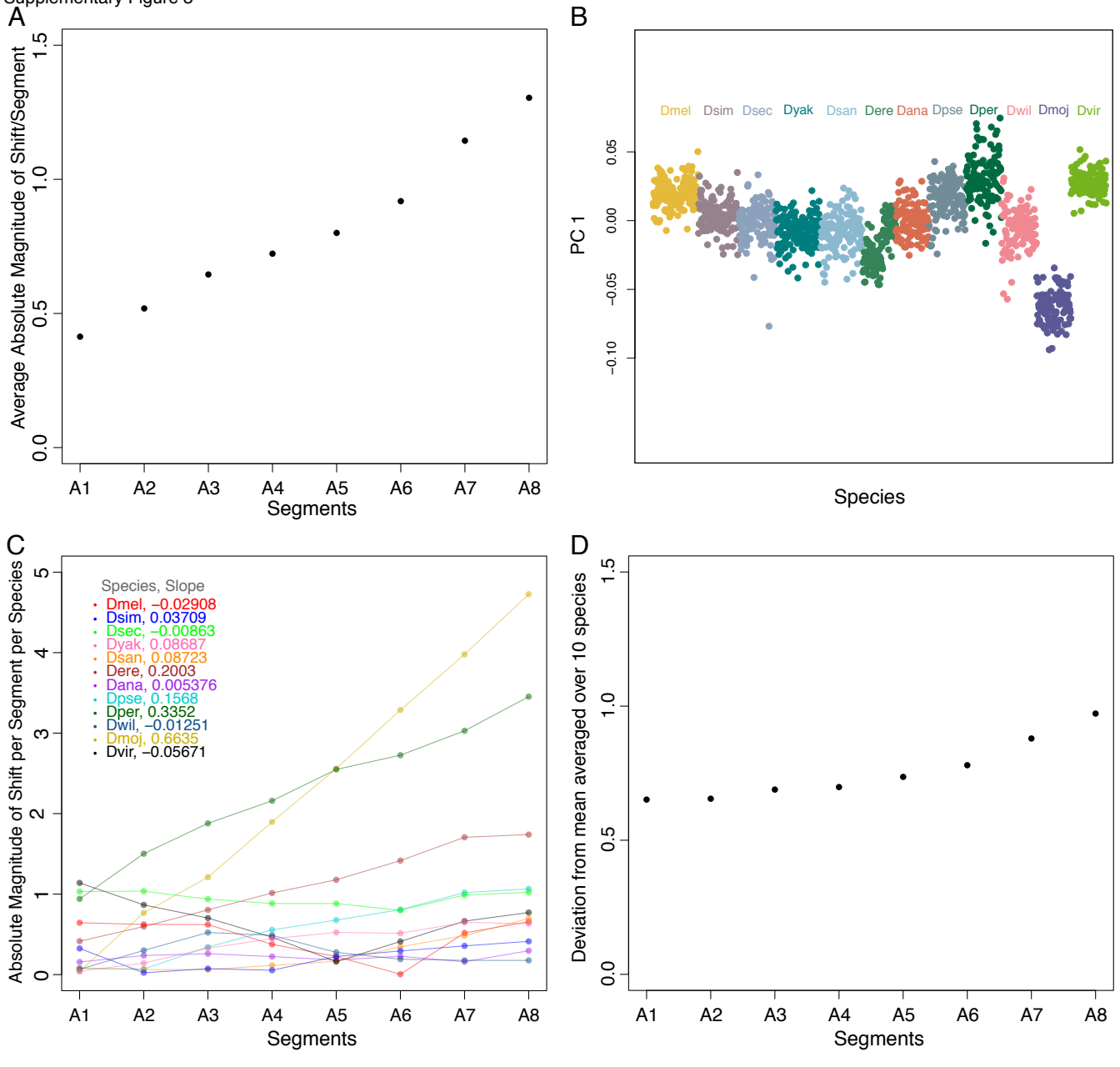
Suplementary Figure 1



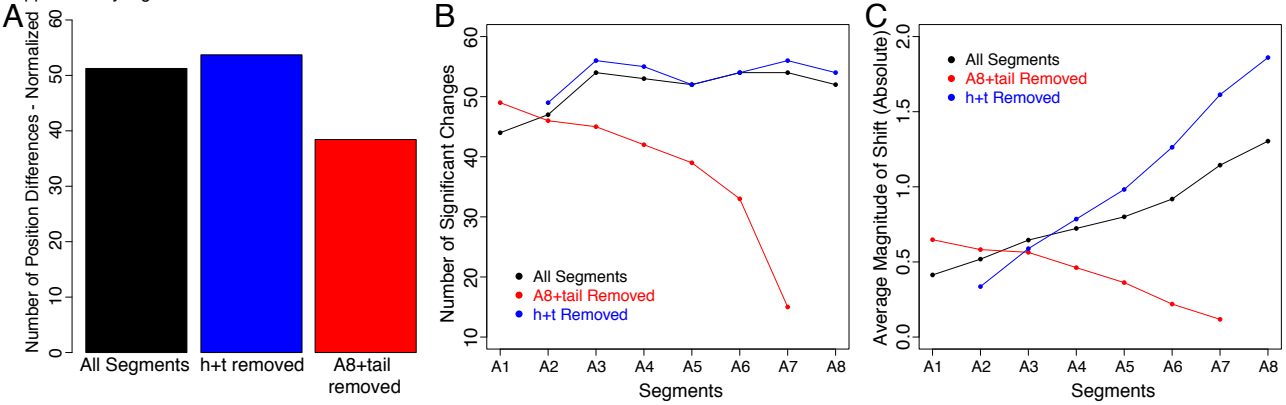
Supplementary Figure 2



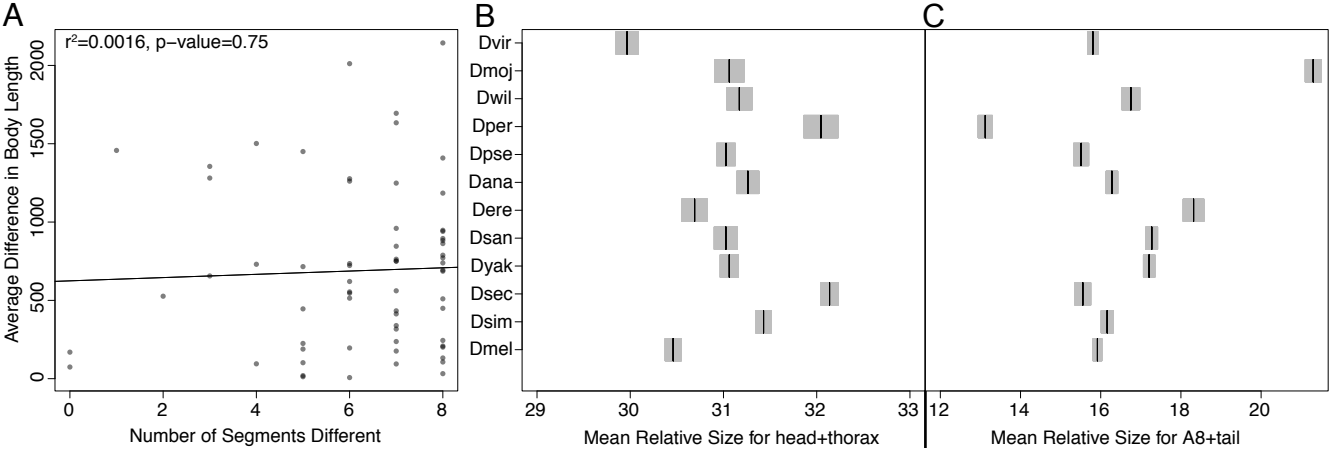
Supplementary Figure 3



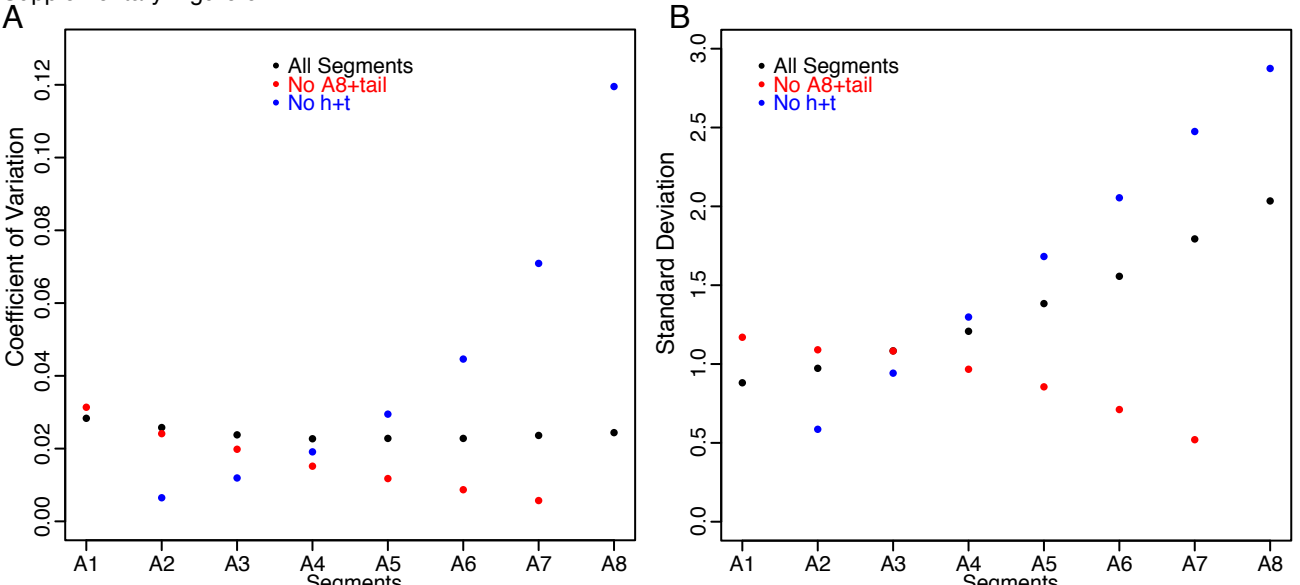
Supplementary Figure 4



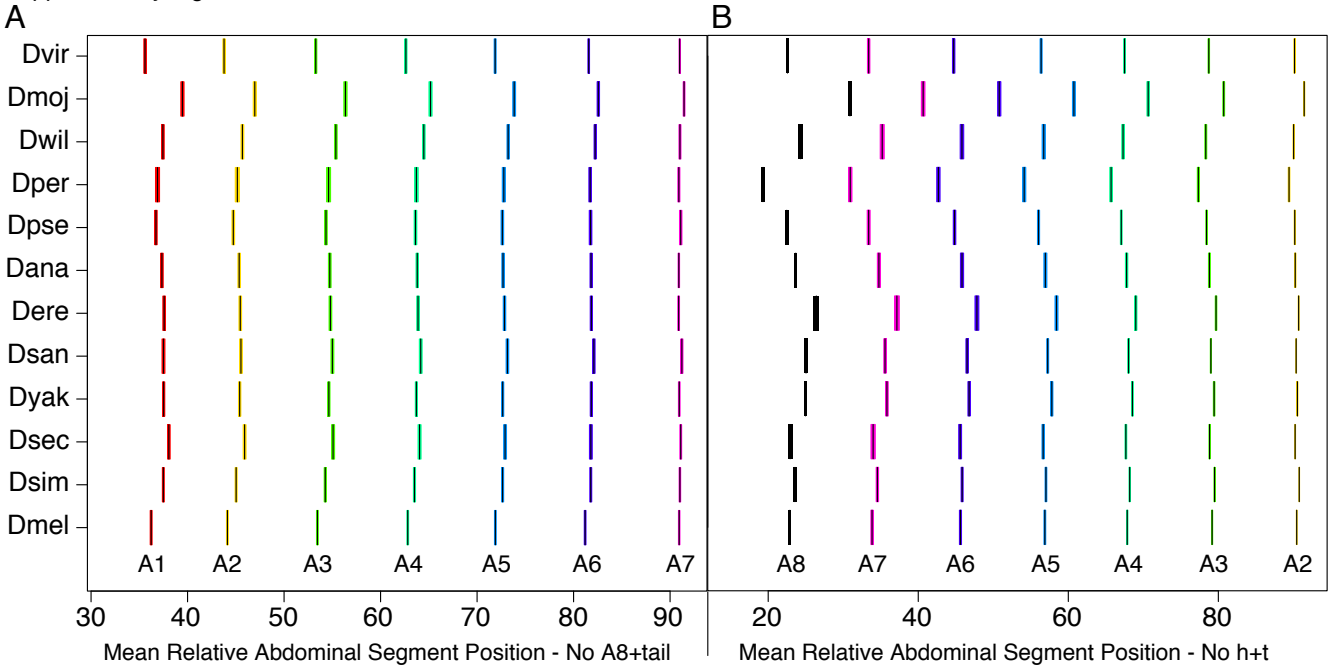
Supplementary Figure 5



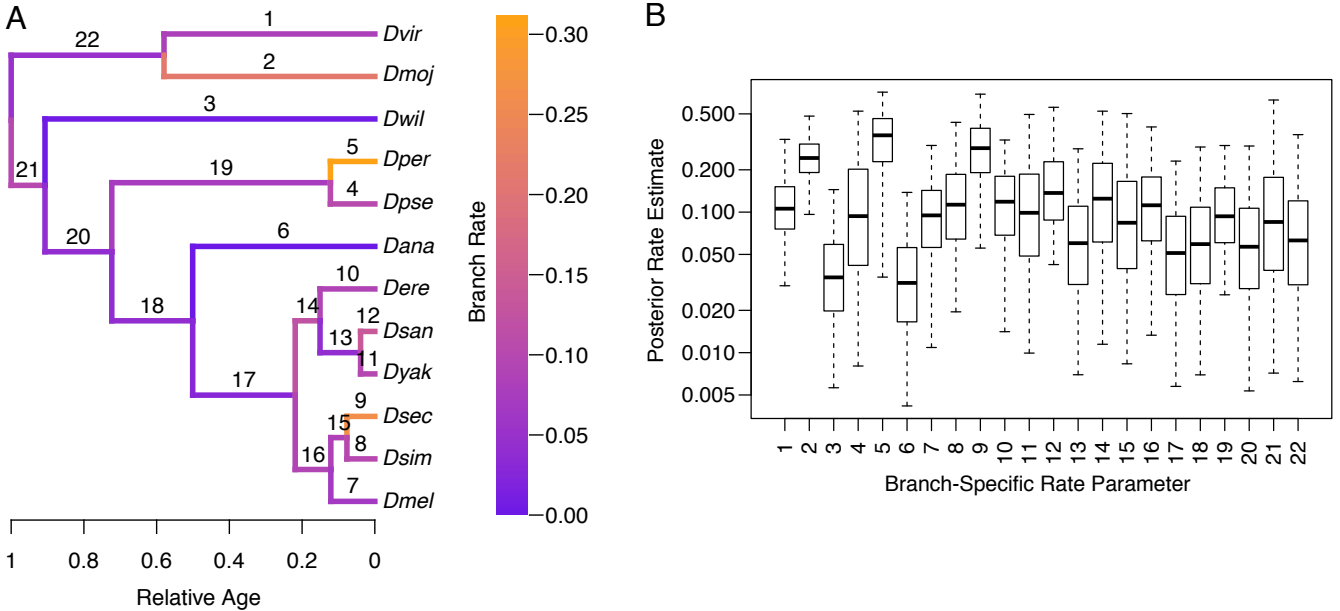
Supplementary Figure 6



Supplementary Figure 7

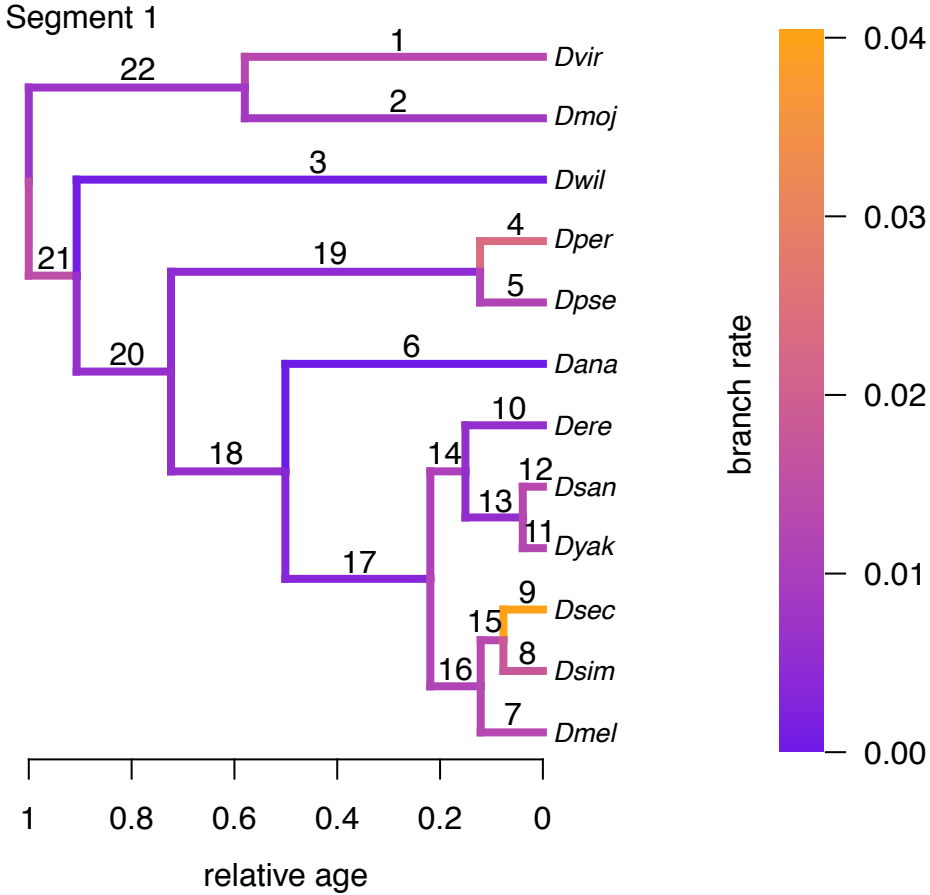


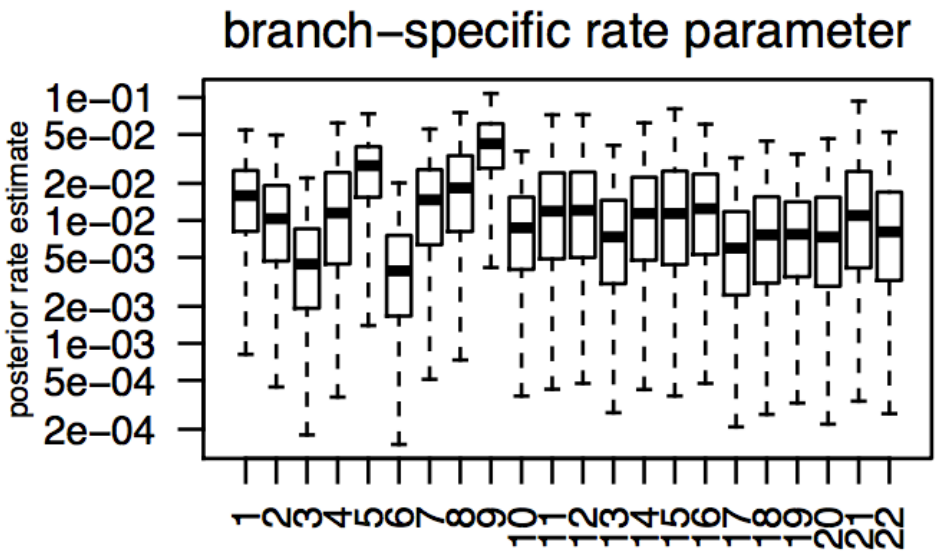
Supplementary Figure 8



Supplementary Figure 9

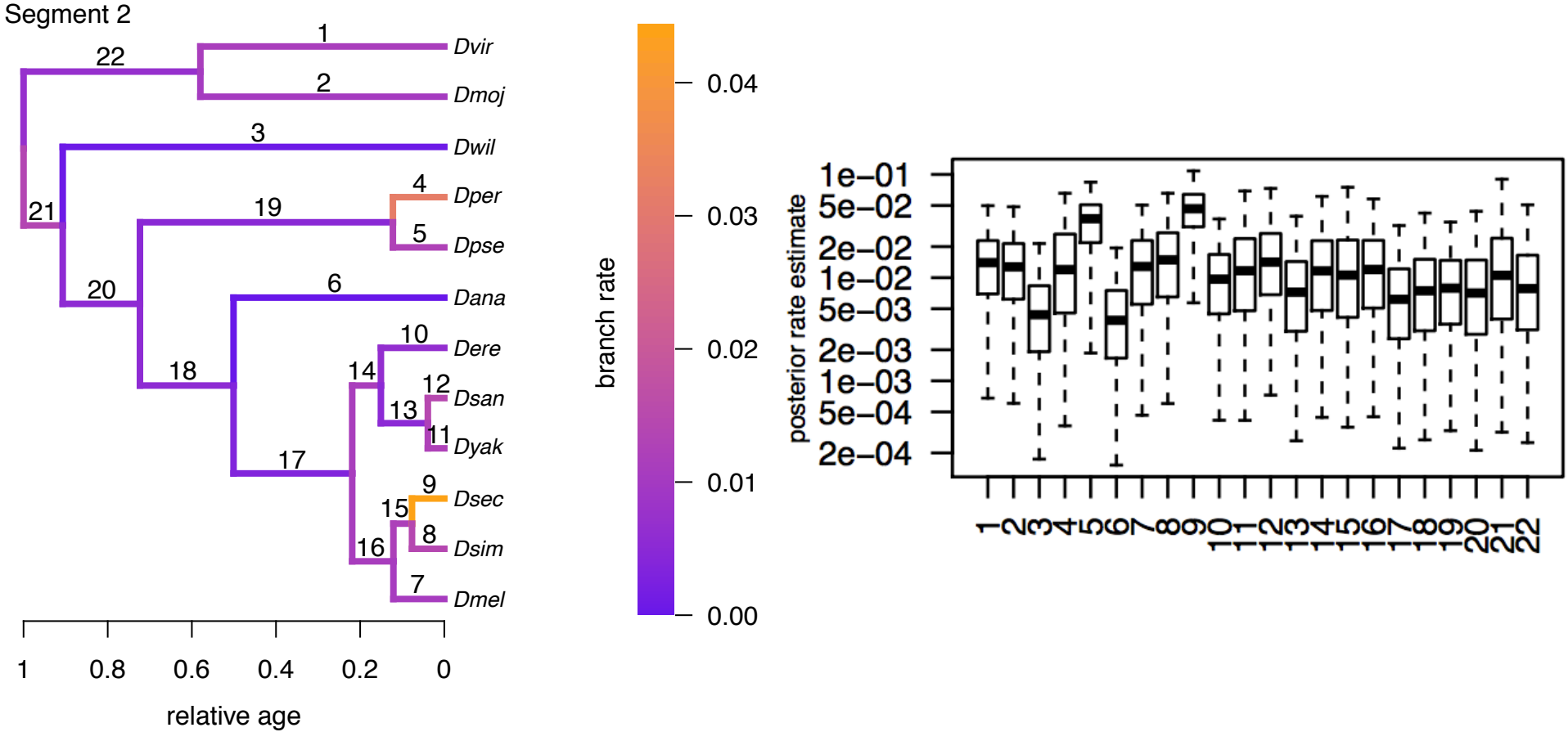
Segment 1





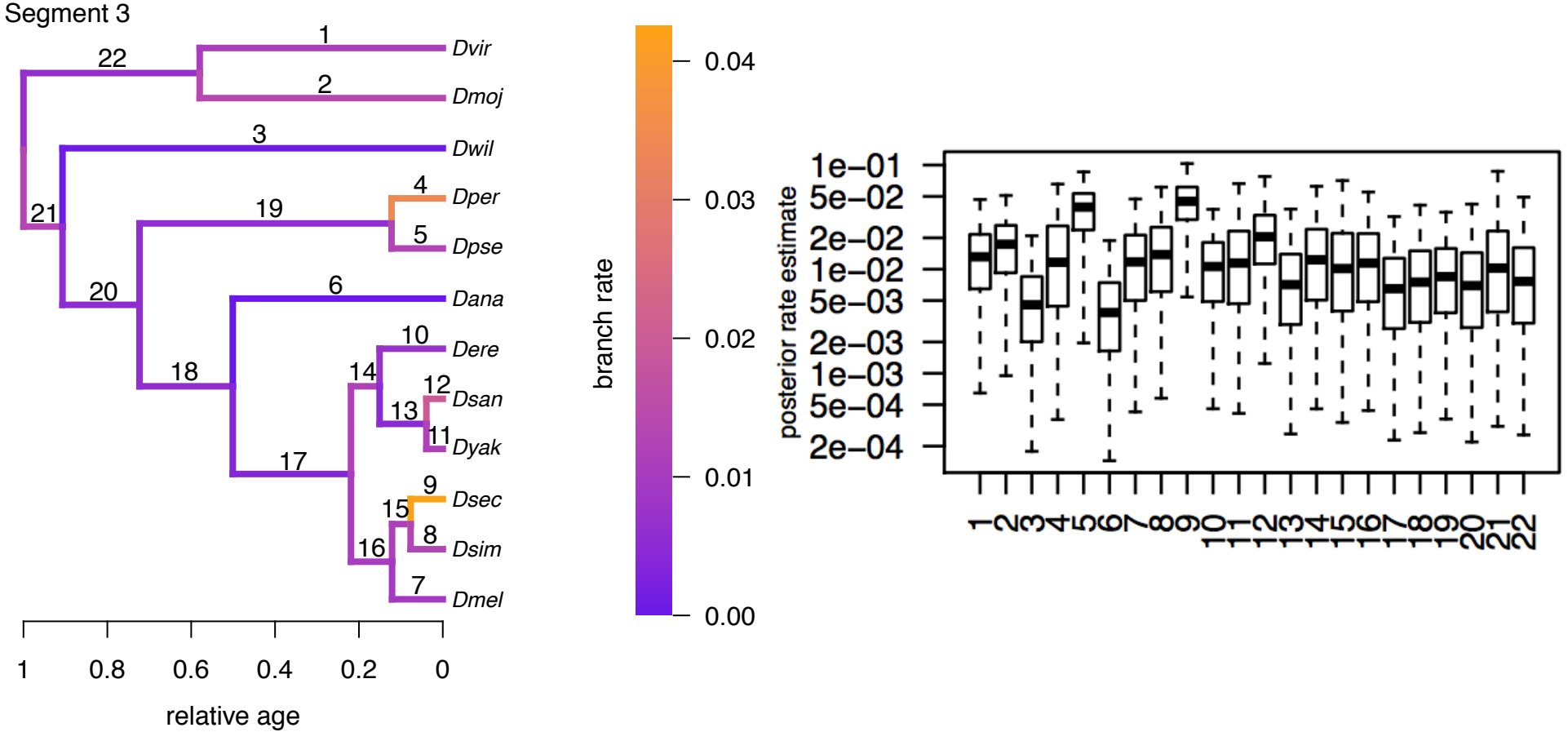
Supplementary Figure 9

Segment 2



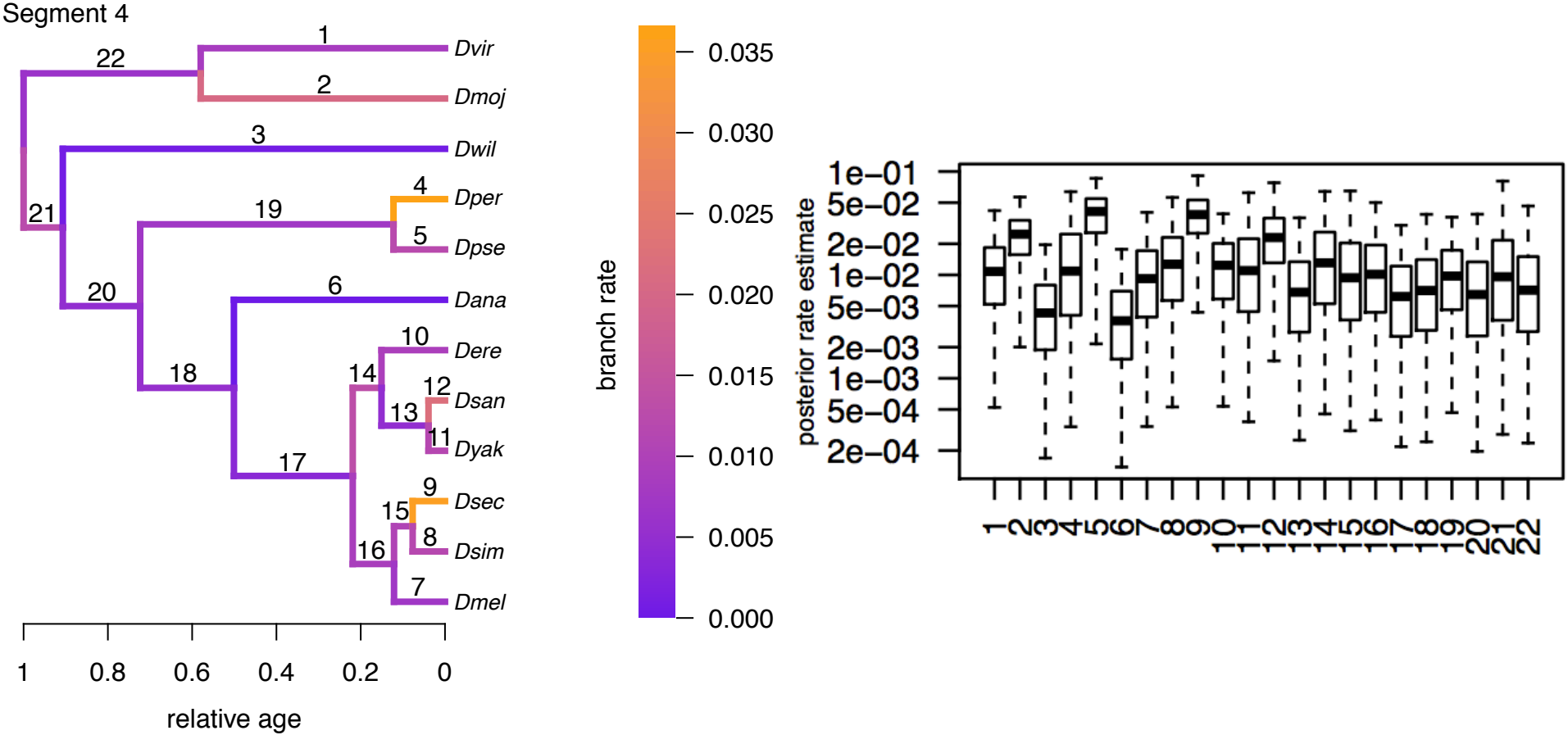
Supplementary Figure 9

Segment 3



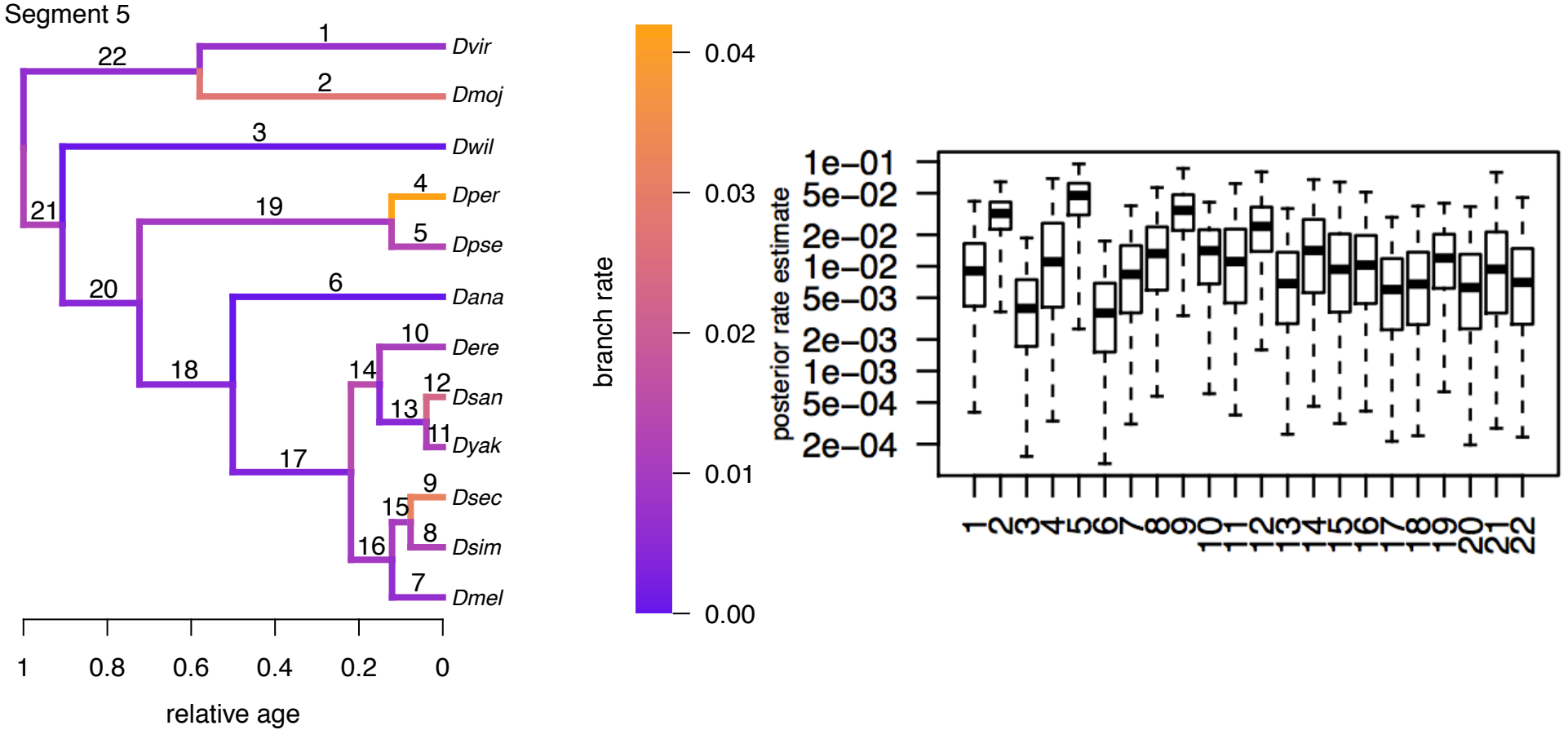
Supplementary Figure 9

Segment 4



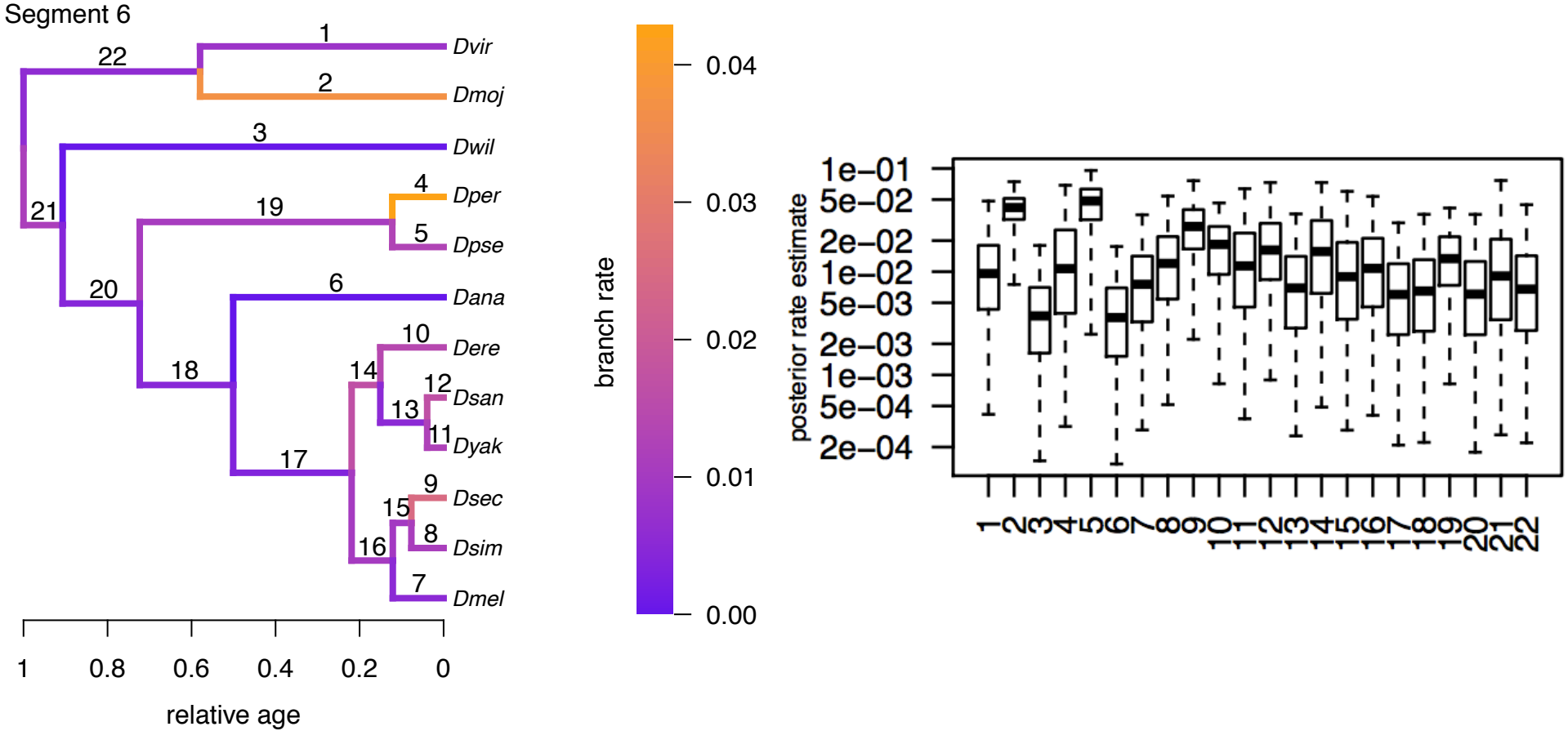
Supplementary Figure 9

Segment 5



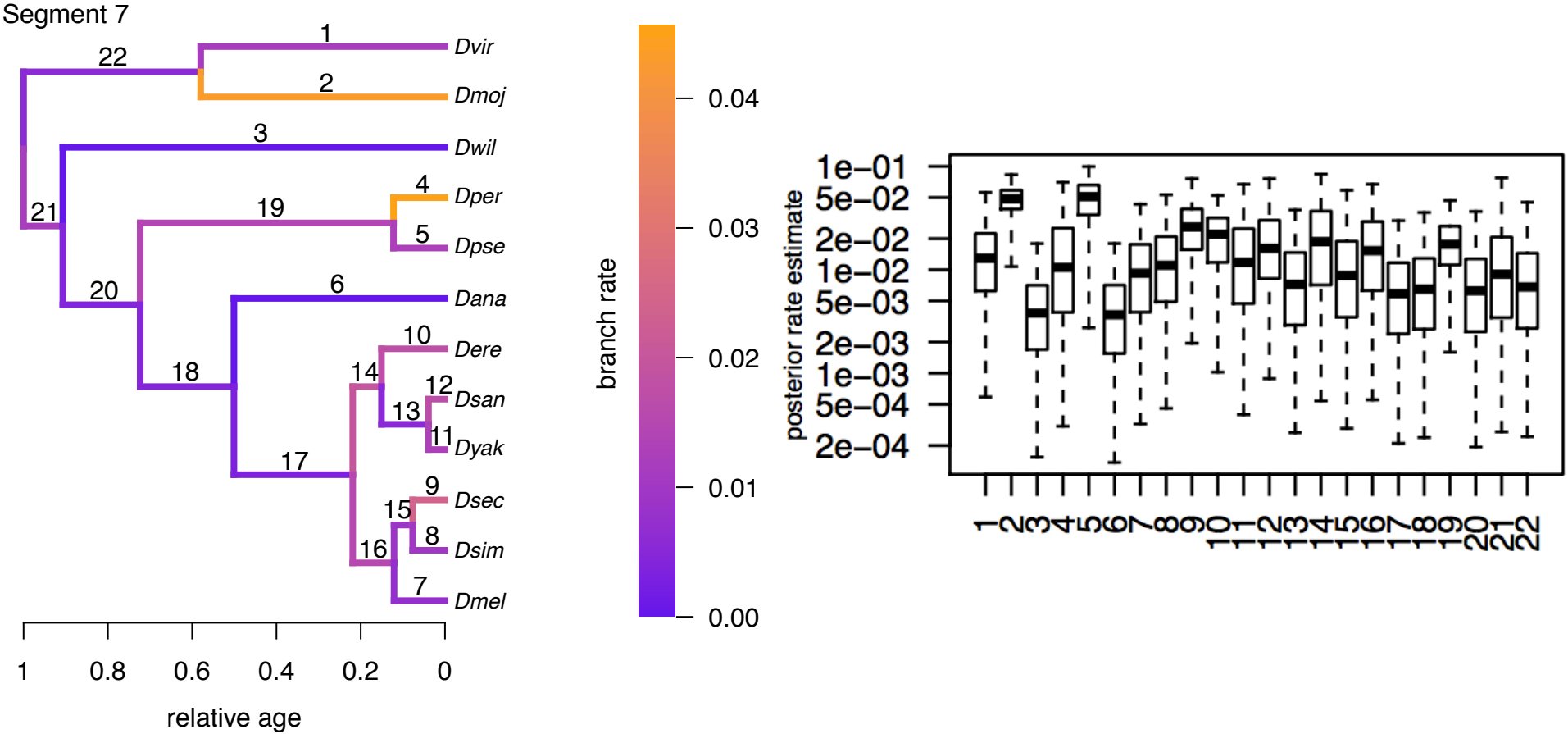
Supplementary Figure 9

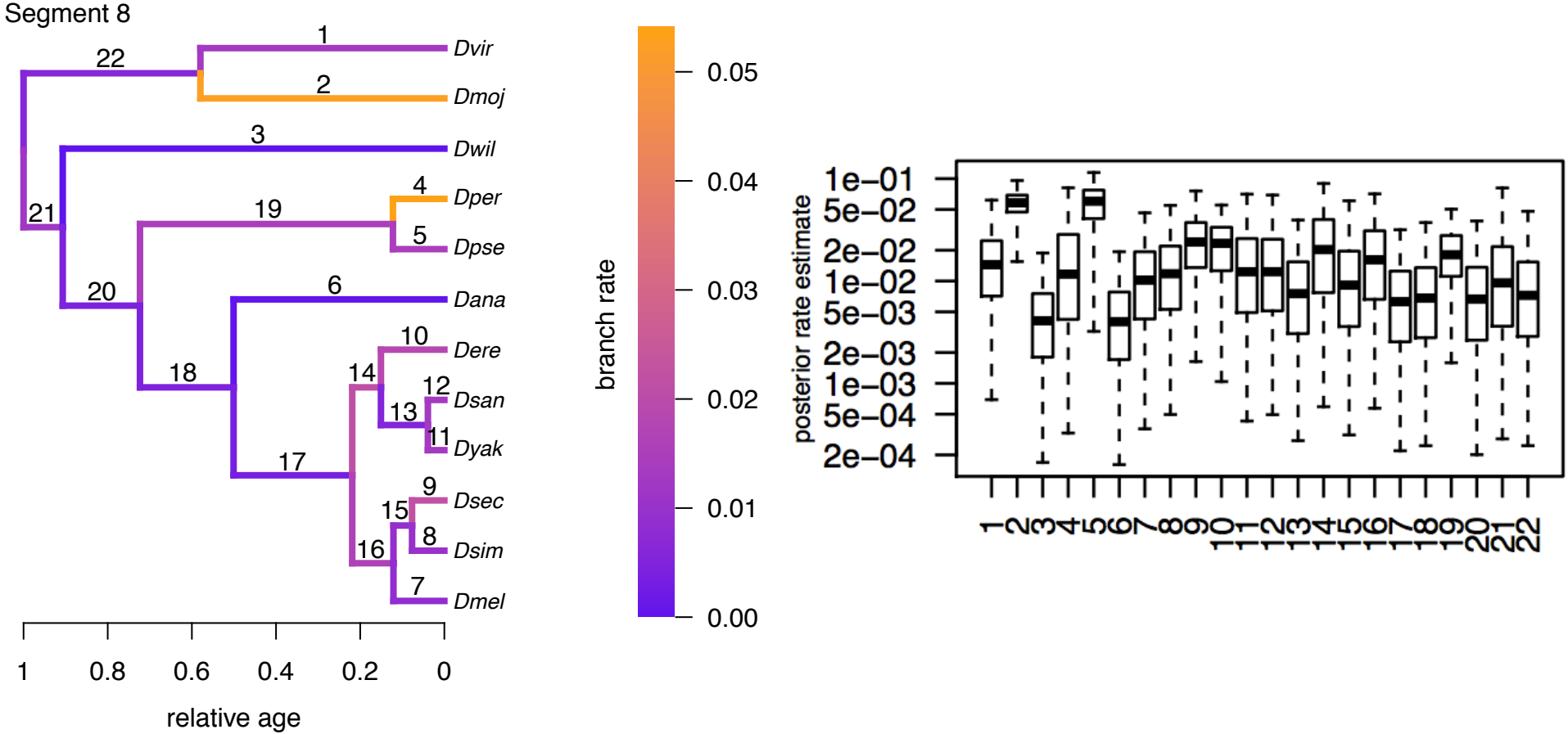
Segment 6



Supplementary Figure 9

Segment 7



Supplementary Figure 9

Segment 8