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1 Title: **Frequent Non-random Shifts in the Temporal Sequence of Developmental Landmark**
2 **Events during Teleost Evolutionary Diversification**
3
4 Running head: **Evolution of teleost developmental sequence**
5
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19 **Abstract**

20 Morphological transformations can be generated by evolutionary changes in the sequence of
21 developmental events. In this study, we examined the evolutionary dynamics of the
22 developmental sequence on a macroevolutionary scale using the teleost. Using the information
23 from previous reports describing the development of 31 species, we extracted the developmental
24 sequences of 19 landmark events involving the formation of phylogenetically conserved body
25 parts; we then inferred ancestral developmental sequences by two different parsimony-based
26 methods—event-pairing (Jeffery, Bininda-Emonds, Coates & Richardson, 2002a) and continuous
27 analysis (Germain & Laurin, 2009). The phylogenetic comparisons of these sequences revealed
28 event-dependent heterogeneity in the frequency of sequence changes. Most of the sequence
29 changes occurred as exchanges of temporally neighboring events. The phylogenetic analyses
30 suggested that the ancestral species had experienced frequent changes in developmental
31 sequences. Although the analyses showed that these heterochronic changes accumulated along
32 phylogenic time, the precise distribution of the changes over the teleost phylogeny remains
33 unclear due to technical limitations. Finally, this first comprehensive analysis of teleost
34 developmental sequences will provide solid ground on which to elucidate the significance of
35 developmental timing in animal morphological diversification.

36

37 **Key Words**

38 Developmental sequence, Evolution, Teleost, Event-pairing, Continuous analysis

39

40

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41 **1. Introduction**

42 Development of multicellular organisms is characterized as a series of morphological events
43 (Smith, 2001). Because individual events progress step by step in a hierarchical manner, one
44 might assume that the temporal sequence of developmental events, i.e., the developmental
45 sequence, does not readily change and is phylogenetically conserved among closely related
46 species that share morphological characteristics. Consequently, if an evolutionary change occurs
47 in the developmental sequence, it could have a significant impact on body patterning and lead to
48 morphological diversity. Indeed, previous comparisons of developmental sequences have
49 revealed rare epoch-making changes that can provide morphological uniqueness to one species
50 (Strauss, 1990; Jeffery, Bininda-Emonds, Coates & Richardson, 2002a; Maxwell, Harrison &
51 Lasson, 2010).

52 Another factor influencing the evolution of developmental sequences is the phylotypic
53 period. This is the developmental period during which evolutionally distant animal species
54 resemble each other. Historically, various models have proposed different time frames for the
55 phylotypic period and discussed why evolutionary changes are difficult to occur in this period
56 (Haeckel, 1874; Duboule, 1994; Raff, 1996; Richardson, 1999). Recently, quantitative
57 approaches have begun to test these models. Transcriptome analyses showed that inter-species
58 diversity is minimum during the middle phase of ontogenetic development (Domazet-Loso &
59 Tautz, 2010; Kalinka et al., 2010; Irie & Kurtani, 2011), supporting the hourglass model that
60 predicts that phenotypic diversity is large in early and late development, and restricted in between.
61 In contrast, quantitative comparison of developmental sequences using 14 vertebrate and
62 mammalian species indicated an opposite trend, namely that phenotypic variation between

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63 species is the highest in the middle phase of development (Bininda-Emond, Jeffery & Richardson,
64 2003). Together with some skepticism surrounding the actual existence of the conserved period
65 (Poe & Wake, 2004; Richardson, 2012), the phylotypic period itself is still a controversial topic in
66 evolutionary developmental biology and requires further rigorous testing with different datasets.

67 Despite the many stimulating theories and ongoing intensive discussion, there is little
68 empirical evidence about evolutionary changes that may have occurred in the developmental
69 sequences; few systematic comparisons have been made of the sequences of a wide range of
70 developmental events covering the entire body in any class of animal. Therefore, there is little
71 data available on how commonly or rarely the developmental sequences had actually changed
72 during evolution. In recent decades, comparative methods to analyze developmental sequences
73 have been developed by several groups (Nunn & Smith, 1998; Jeffery et al., 2002a; Jeffery,
74 Richardson, Coates & Bininda-Emonds, 2002b; Jeffery, Bininda-Emonds, Coates & Richardson,
75 2005; Harrison & Larsson, 2008; Germain & Laurin, 2009). These methods compare the relative
76 order of developmental events among different species and estimate potential evolutionary shifts
77 of the events, i.e., "heterochronic shifts", in a parsimonious manner (Schoch, 2006; Smirthwaitet,
78 Rundle, Bininda-Emonds & Spicer, 2007; Sanchez-Villagra, Goswami, Weisbecker, Mock &
79 Kuratani, 2008; Hautier et al., 2013; Laurin, 2014; Werneburg & Sanchez-Villagra 2015; Carril
80 & Tambussi, 2017). Although most previous analyses focused on the developmental sequences
81 for a specific organ or limited body part, we considered the methods themselves to be similarly
82 applicable to a global analysis of developmental events comprising the entire body.

83 In this study, we conducted the first comprehensive survey of developmental sequences
84 of teleosts. Teleosts comprise numerous species characterized by great morphological diversity

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85 (Nelson, Grande & Wilson, 2016) and shared conserved body structures including vertebrae, eyes,
86 and paired fins (Romer & Parsons, 1986). Owing to the popularity of some teleosts as
87 developmental research models, there are well-established staging tables for many teleost species
88 covering common clear-cut developmental landmarks. Hence, the teleost can provide an ideal
89 dataset for systematic analysis of developmental sequences. Among the widely used
90 developmental landmarks, we chose 19 events that individually contribute to distinct body parts
91 across the whole body. Using the dataset of 31 actinopterygian species, we compared the
92 developmental sequences and reconstructed the ancestral sequences over the teleost phylogeny.
93 Our analysis indicates that the developmental sequences have changed frequently during
94 evolution. However, the changes were not random; the order of a few events seemed to have
95 shifted more frequently than the others in the developmental sequence. These developmental
96 sequence rearrangements may have contributed to shaping teleost morphological diversity.
97

98 **2. Materials and Methods**

99 **2.1 Construction of the teleost phylogenetic tree**

100 The overall topology of the phylogenetic tree followed the molecular phylogenetic relationship
101 previously reported by Near et al. (2012, 2013). The minor branches missing in the tree were
102 inserted based on the phylogenetic data obtained from Saitoh et al. (2011) and Yang et al. (2015)
103 for Cypriniformes, Perez et al. (2007) and Friedman et al. (2013) for Cichliformes, and Pohl,
104 Milvertz, Meyer & Vences (2015) for Cyprinodontiformes. The divergence times were
105 determined using the public database TIMETREE, *The Timescale of Life* (Hedes & Kumar, 2009)
106 (Supplemental file 1).

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107

108 **2.2 Data sampling**

109 Information about developmental events was extracted from 31 published research articles that
110 describe normal development of actinopterygian species (Figure 1). These developmental events
111 were selected according to the following criteria: (1) clearly defined in most of the selected
112 articles, (2) involved in a wide range of body parts and systems, and (3) occurring from early to
113 late phases of embryogenesis. The 19 events examined in the study were the first instances of
114 blood circulation (bc), caudal fin rays (cfr), eye pigmentation (ep), embryonic shield (es), the first
115 somite (fs), hatching (h), heart beating/pulsing (hb), Kupffer's vesicle (kv), lenses or lens
116 placodes (le), a medial finfold (mff), a mouth opening (mo), olfactory vesicles/pits/placodes (olf),
117 otoliths (oto), otic vesicles/placodes/primordia (ot), optic vesicles/placodes/primordia (op),
118 pectoral fin buds (pfb), a tail bud (tb), three brain regionalization (tbr), and tail lift from the yolk
119 (tl). The temporal order of the developmental events was ranked according to descriptions in the
120 articles (Figure 1). When an article did not describe a developmental event, the event was treated
121 as a missing datum.

122

123 **2.3 Relative scaling of the ranks of developmental events**

124 The raw ranks of individual developmental events were determined from the developmental
125 sequences of extant species (Figure 1). When multiple events were rated as occurring
126 simultaneously, the same rank numbers were replaced by the average rank. The ranks in each
127 sequence were then normalized to the relative scaling of 0 to 1, so that "0" was assigned to the

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128 lowest rank and "1" to the highest rank (Germain & Laurin, 2009). Similarly, normalized ranks
129 were calculated for ancestral developmental sequences reconstructed as described below.
130

131 **2.4 Reconstruction of ancestral developmental sequences**

132 To reconstruct ancestral developmental sequences, we used two different methods: event-pairing
133 (Jeffery et al., 2002a) and continuous analysis (Germain & Laurin, 2009).

134 When event-pairing (Jeffery et al., 2002a) was used to infer ancestral sequences, all
135 171 pairs (19×18/2) of developmental events in each species were scored based on the relative
136 timing; when one event occurred earlier, simultaneously, or later than another event, the timing
137 was rated as 0, 1, or 2, respectively. By comparing the scored event-pairing matrices of different
138 species, the ancestral event-pairing matrix was reconstructed at each node of the teleost
139 phylogenetic tree in a parsimonious manner under accelerated transformation (acctran) and
140 delayed transformation (deltran) optimizations using PAUP* software (Swofford, 2002). The
141 ancestral event-pairing matrices were then reconverted to ancestral developmental sequences
142 (Supplemental file 2).

143 In the continuous analysis (Germain & Laurin, 2009), the normalized ranks for each
144 event calculated as described above were used as a continuous time scale to estimate the timing of
145 the event in a hypothetical ancestor by squared-change parsimony (Maddison, 1991). The ranks
146 of individual events in the hypothetical ancestors were estimated using the PDAP module of the
147 Mesquite software (Maddison & Maddison, 2002, Midford, Garland & Maddison, 2003) (Figure
148 S1-S19). According to the ranks, the events were then arranged into the ancestral developmental
149 sequences (Supplemental file 2).

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150

151 **2.5 Quantification of rank variation for individual developmental events**

152 To quantify rank variation for each event, the pairwise distance in the normalized ranks of an
153 event was calculated by comparing the sequence on a branch to its immediate ancestor in the
154 teleost phylogenic tree. The distances obtained from all the branches were then summed and
155 averaged for the number of branches examined. The missing events in some branches were
156 excluded from the calculation. The rates of evolution for developmental events by continuous
157 analysis were obtained using the Mesquite PDAP module (Maddison & Maddison, 2002;
158 Midford et al., 2003).

159

160 **2.6 Detection of heterochronic shifts using a Parsimov algorithm**

161 The heterochronic shifts between two developmental sequences at each phylogenetic node were
162 estimated using the Parsimov algorithm developed by Jeffery et al. (2005). This parsimony-based
163 algorithm determines the minimum number of event shifts that can explain the difference
164 between two developmental sequences. Following the instructions, we implemented a Perl script,
165 Parsimv7g.pl, with PAUP* output log file under both acctran and deltran optimizations, and
166 mapped the detected heterochronic shifts onto the teleost phylogeny (Supplemental file 3).

167

168 **2.7 Simulation of the reference distribution of heterochronic shifts**

169 A reference distribution of heterochronic shifts over the teleost phylogeny was created by
170 computer simulation based on a simple null assumption that a heterochronic shift occurs at a
171 constant rate per unit time and accumulates in proportion to branch length. In this simulation, we

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172 did not consider the event-dependent differences in the shift frequencies. The simulation
173 distributed the estimated number of heterochronic shifts randomly over the teleost phylogenic
174 branches depending solely on their branch lengths. The simulation was replicated 100,000 times
175 to obtain a reference null distribution. The null distribution of heterochronic shifts was then
176 compared to the distribution of heterochronic shifts estimated from experimental datasets.
177

178 **2.8 Calculation of total rank changes in individual phylogenic branches**

179 The pairwise distance in the normalized ranks for each event was calculated as described above.
180 The distances for all the events were then summed for each branch. The calculated values were
181 then normalized by dividing by the number of examined events (excluding the missing event),
182 and represented as the total rank changes per branch (Supplemental file 4).
183

184 **3. Results**

185 **3.1 Phylogenetic relationship of the 31 actinopterygian species**

186 For the present analyses, we selected 30 teleost species belonging to 13 distinct orders as the
187 in-group because their developmental sequences have been well-documented (Figure 1). As an
188 out-group, we used the amiadae fish, *Amia calva*, as a recent molecular genetic analysis had
189 confirmed its location as the out-group of the teleost (Near et al., 2012); furthermore, it retains
190 ancestral morphological characteristics such as mineralized scales, gas bladder lung, and
191 heterocercal tail (Nelson, Grande & Wilson, 2016). In the constructed phylogenetic tree, the
192 examined species were widely distributed and represented distinct branches of the teleost clade
193 (Figure 2). Because there are very few reports on fish development in a marine environment, the

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194 species covered in this study were basically freshwater teleosts; however, several anadromous
195 species were also included such as *Gasterosteus aculeatus*, which develops in freshwater but
196 alternates between the sea and freshwater as an adult.
197
198 **3.2 Comparison of temporal orders of developmental events among teleost species**
199 We selected 19 developmental events that appeared consistently as landmarks in the
200 developmental staging of many teleost species (Figure 1). To gain an overall view of
201 developmental sequences involved in the formation of entire body, we intentionally included
202 events that belong to substantially different biological systems and contexts such as those
203 originating from different germ layers or giving rise to different cell types or separate body parts.
204 Additionally, the list also included a small number of interrelated events such as formation of the
205 optic vesicles/placodes/primordia (op), lenses or lens placodes (le), and eye pigmentation (ep).
206 We gathered information on these 19 events from articles reporting the development of 31
207 actinopterygian species, and ranked the orders of individual events in the temporal sequence for
208 each species (Figure 1).
209 We first compared rank orders of individual events among 30 in-group teleost species.
210 To minimize effects of simultaneous occurrence of events and missing data, the raw ranks (Figure
211 1) were rescaled to the normalized ranks that fit within the same range, from 0 to 1, in all the
212 teleost species (see Methods). Figure 3a shows the distribution of the normalized ranks for
213 individual developmental events, which were horizontally arranged according to the average
214 values. Interestingly, the ranges of variation in the ranks differed widely, depending on the event.
215 One extreme case was embryonic shield (es), which always appeared first and with no variation in

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216 the developmental sequences obtained from the 29 teleost species, excluding one missing
217 description for *Galaxias maculatus* (Figure 1). In contrast, relatively large variations in the rank
218 were observed for the appearance of olfactory vesicles/pits/primordia (olf) and medial finfold
219 (mff), suggesting that the temporal order of these events in the developmental sequence can
220 change more easily (Figure 3a).

221 To explore the evolutionary history of developmental sequences, we next estimated
222 ancestral developmental sequences on each phylogenetic branch using the event-pairing method
223 (Jeffery et al., 2002a). This algorithm compares the relative orders of all the event pairs between
224 two different developmental sequences and generates the ancestral sequences determined as a
225 parsimonious solution under acctran and deltran optimizations (Supplemental file 2). Using the
226 obtained ancestral developmental sequences, we compared the normalized ranks of individual
227 events as in Figure 3a. Overall, the rank orders of individual events in the ancestral developmental
228 sequences (Figure 3b and 3c) were similar to the extant sequences (Figure 3a). When the
229 developmental events were horizontally aligned in the same order as the extant average ranks,
230 there were only a few inversions in the order of two successive events at the average level (e.g.,
231 the order between first somite [fs] and tail bud [tb]). The range of variation for individual events
232 was also similar to the extant sequences, further confirming that the ranks of some developmental
233 events change more frequently than others during evolution.

234 We repeated the same rank analyses using ancestral developmental sequences inferred
235 by a different method developed by Germain & Laurin (2009). This “continuous analysis” uses
236 the normalized rank of events as a continuous ontogenetic time scale and directly estimates the
237 timing of individual events that should occur in hypothetical ancestors based on squared-change

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238 parsimony (Maddison, 1991). Because the two methods are based on different assumptions, they
239 produced significantly different ancestral sequences (Supplemental file 2). The rank variations of
240 the sequences showed a similar but slightly different trend, namely that the ranks for appearance
241 of otic vesicles/placodes/primordia (ot) and olfactory vesicles/pits/primordia (olf) were relatively
242 highly variable (Figure 3d).
243

244 **3.3 Evaluation of rank changes through the evolutionary history of the teleost**

245 Because rank variations fluctuate depending on the event, we next evaluated rank changeability
246 phylogenetically along the course of teleost evolution. We measured the pairwise rank distance
247 between a sequence and its immediate ancestral sequence across the node in the teleost
248 phylogenic tree (Supplemental file 4), represented as the average value per branch (Figure 4a).
249 For this analysis, ancestral developmental sequences were obtained by event-pairing (Jeffery et
250 al., 2002a) under acctran and deltran optimizations. The index scores were highly consistent
251 regardless of the optimization method used (Spearman's rank correlation for the two
252 optimizations; r = 0.7621). When the events were chronologically arranged along the standard
253 ontogenic time frame, defined as the average rank orders in the extant teleosts, the overall shape
254 of the ontogenic histogram did not show any clear characteristics in the middle phase of the
255 developmental sequence characterized by three brain regionalization (tbr), otic
256 vesicles/placodes/primordia (ot), and lenses or lens placodes (le). This developmental term
257 typically corresponds to the narrow neck in the hourglass model. The ontogenic histogram was
258 instead relatively skewed toward the late tail bud stage involving olfactory
259 vesicles/pits/primordia (olf) and otoliths (oto). Because the late tail bud stage also included

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260 low-profile events such as blood circulation (bc), we do not consider the weak upward trend as a
261 continual ontogenic pattern formed by temporally clustered events.
262 We also examined evolutionary rank changes using a different index. Based on
263 continuous analysis (Germain & Laurin, 2009), the evolutionary rate of rank changes can be
264 obtained for each event (Figure 4b). This index shows rank changeability per million years and
265 therefore differs slightly from the one per branch used in the previous analysis. The calculation
266 also involved the set of different ancestral sequences estimated by continuous analysis.
267 Nevertheless, this new index was basically similar to the previous analysis, with the olfactory
268 vesicles/pits/primordia (olf) exhibiting high rank changeability. In the ontogenic sequence, this
269 high score event was like an independent peak with no indication of a global ontogenic pattern.
270 These and previous results further suggest that the ranks of olfactory vesicles/pits/primordia (olf)
271 have changed more frequently during evolution than the other events.
272

273 **3.4 Event exchange rates in developmental sequences**

274 We next focused on the actual sequence order of developmental events. Figure 5 shows the
275 percentage of sequences in which one event (shown in the row) occurs later than another (shown
276 in the column) among the 30 extant in-group species. In general, the sequence of two temporally
277 distant events was somewhat conservative, with no order reversal with many combinations,
278 whereas the orders of neighboring events changed more frequently. Closer inspection of
279 anatomically interrelated events indicates that the temporal order of the optic
280 vesicles/placodes/primordia (op) and the lenses or lens placodes (le) was fixed in all the species,
281 and that of the lenses or lens placodes (le) and the eye pigmentation (ep) was practically fixed,

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282 except for one sequence reversal in *Heterobranchus bidorsalis*. Similar results were obtained
283 from the comparison of event orders in the ancestral developmental sequences (Figure S20a and
284 S20b).
285

286 **3.5 Distribution of heterochronic shifts across the teleost phylogenetic tree**

287 Using event-pairing with a Parsimov algorithm (Jeffery et al., 2005), we next searched for
288 heterochronic shifts of the events that can explain the changes from one sequence to another at
289 every node of the teleost phylogenetic tree. Although this is a parsimony-based algorithm and,
290 therefore, estimates the minimum number of event shifts, we obtained 167 (acctran), 162 (deltran),
291 and 91 (conserved between acctran and deltran) heterochronic shifts in total (Supplemental file 3).
292 The teleost phylogenetic tree has 59 branches in total and when the estimated shifts were mapped
293 onto the tree, multiple heterochronic shifts were observed in almost all the branches
294 (Supplemental file 3).

295 Because a substantial number of heterochronic shifts were inferred, we examined the
296 distribution of these shifts in the teleost phylogeny. Based on the simple null hypothesis that a
297 heterochronic shift occurs at a stochastically random manner and accumulates neutrally and
298 exponentially along phylogenic time, we calculated a reference distribution of heterochronic
299 shifts by computer simulation (white circles in Figure 6a and 6b). Compared to this reference null
300 distribution, the actual heterochronic shifts inferred by the Parsimov algorithm exhibited a
301 somewhat enigmatic distribution, with a constant number of shifts relative to branch length with
302 both acctran and deltran optimizations (black circles in Figure 6a and 6b). The number of
303 heterochronic shifts per branch showed a smaller coefficient of variation compared to the

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304 reference value (Figure 6c), indicating that branch-by-branch fluctuations in the number of
305 heterochronic shifts were more limited than those estimated from random allocation of the shifts.
306 The constant trend in the shift number was also supported by the fact that the number of
307 phylogenetic branches harboring no heterochronic shifts from the experimental dataset was
308 significantly smaller than the reference data (Figure 6d). Because inclusion of an extremely long
309 branch could skew the statistical results, we performed the same statistical comparison but using
310 only relatively short branches (≤ 50 Mya and ≤ 20 Mya in Figure 6c, 6d). We also replaced the
311 phylogenic time scale with the generation number (Figures S21 and S22) by considering the
312 average generation time of individual species (Supplemental file 5). However, these
313 modifications did not qualitatively change the results of the analysis.

314 A potential caveat about standard parsimony is that it may give ambiguous solutions as
315 equally likely by considering only the number of changes, and not the branch length. Thus, we
316 conducted a similar analysis of heterochronic rank changes using the ancestral developmental
317 sequences inferred by continuous analysis, a modified parsimony method that incorporates
318 branch length information to find a single most likely solution (Germain & Laurin, 2009). When
319 we used the ancestral sequences inferred by standard parsimony-based event-pairing, the
320 calculated total rank changes involving all the events per branch exhibited a constant trend
321 against the branch length (Figure 7a and 7b, Supplemental file 4), similar to the preceding
322 analysis. The Spearman's rank correlation coefficients with regard to branch length showed a
323 marginally significant, but weak correlation (acctran: 0.3040, deltran: 0.3428). When the
324 ancestral sequences inferred by continuous analysis were used instead for the calculation, the
325 total rank changes presented a stronger correlation with the branch length, showing a clear

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326 proportional increase (Figure 7c, Spearman's rank correlation coefficient: 0.5723). These results
327 imply that the selection of ancestral sequences has a strong influence in this type of analysis,
328 and that the enigmatic distribution of heterochronic event shifts observed with standard
329 parsimony can be corrected by allowing for the effect of phylogenetic time, leaving the
330 distribution of event shifts still unclear. A more important indication from the comparisons may
331 be that similar numbers of heterochronic event changes were estimated, regardless of the
332 method (Figure 7d and 7e). These numbers, which are parsimonious estimates, further support
333 that heterochronic shifts of developmental events were frequent during teleost evolution.
334

335 **4. Discussion**

336 In the present study, we systematically analyzed evolutionary changes in the developmental
337 sequences of teleosts using a wide range of species and developmental events. The results
338 revealed how teleost ancestors experienced dynamic and frequent rearrangements of
339 developmental sequences leading up to extant species. This first comprehensive analysis of
340 teleost developmental sequences will provide a valuable foundation toward understanding the
341 significance of evolutionary changes in developmental sequences for animal morphological
342 diversification.

343 Initially, we expected a more conservative evolution of developmental sequences
344 because we only examined highly conserved traits in the phylogeny and species belonging to a
345 restricted infraclass. However, we found more frequent heterochronic changes than expected,
346 leading to very weak phylogenetic signals. This may be due to polymorphic fluctuations in
347 developmental timing, observable even in a single species (de Jong, Colbert, Witte &

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348 Richardson, 2009; Mitgutsch, Wimmer, Sanchez-Villagra, Hahnloser & Schneider, 2011). If the
349 developmental timing is not fixed, comparisons of different species staged under different
350 conditions should further increase false positive signals (Weisbecker & Mitgutsch, 2010). In
351 addition, our wide selection of developmental events may have influenced the results; in this
352 study, we intentionally adopted events covering a variety of embryonic origins, cell types, body
353 parts, or biological systems to understand global body patterning. In contrast, several previous
354 studies aimed for in-depth understanding the developmental sequences for a specific body part
355 or organ (Schlosser, 2008; Hautier et al., 2011). For example, in the comparative analysis of
356 neural development in 18 mammalian species, the whole sequence of 271 neural developmental
357 events was strikingly preserved, even though many of the events occur in separate places
358 (Workman, Charvet, Clancy, Darlington & Finlay, 2013). The ossification sequence of cranial
359 bones in early mammals was also seen to be highly conserved (Sanchez-Villagra et al., 2008). It
360 is conceivable that development of individual elements in the same tissue or organ is more
361 hierarchically constrained, whereas different tissues or body parts may have more freedom to
362 behave as independent modules during development (Klingenberg, Badyaev, Sowry &
363 Beckwith 2001; Schmidt & Starck, 2010; Kawanishi et al., 2013; Laurin, 2014). The modular
364 nature of individual body parts may explain why relatively frequent changes of developmental
365 sequences were observed in this study, and may possibly serve as a source for the independent
366 evolution of different body parts.

367 Another important note from the present study is that heterochronic shifts were frequent
368 but not random. The rank analyses indicated that the temporal orders of some developmental
369 events change more drastically than others during evolution. Although the magnitude fluctuates

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370 according to the method, the olfactory vesicles/pits/primordia (olf) consistently exhibited high
371 rank changeability in all the analyses. The olfaction is a critical sensor for environment
372 monitoring in animals. Conceivably, ancestral teleosts could have benefited from temporally
373 accelerating or decelerating the formation of the olfactory sensory organs for better adaptation
374 to environmental conditions.

375 Regarding the phylotypic period, the present study did detect neither a significant
376 signature of evolutionary restriction of ontogenic event changes, nor an inverted increasing
377 trend of heterochronic shifts during the middle phase of development, as previously reported
378 (Bininda-Emond et al., 2003). Overall, we did not observe ontogenically clustered behavior of
379 developmental events. Conceptually, our finding is close to the conclusion of Poe & Wake
380 (2004): the simple rule that the orders of temporally neighboring events change more frequently
381 is more relevant than other complicated models. Our result is important because we used
382 different datasets for this analysis.

383 Although maximum parsimony has been a widely used criterion in evolutionary studies,
384 it contains various drawbacks (Germain & Laurin, 2009). One drawback in the present study
385 was that it considers only the number of character changes, and not phylogenetic time, to
386 calculate the ancestral sequence. For example, under maximum parsimony, a single change on
387 one short internal branch is always chosen as the realistic scenario compared to two parallel
388 changes on two long external branches (Felsenstein, 1978). We obtained a somewhat enigmatic
389 distribution of heterochronic shifts using the sequences inferred by the standard
390 parsimony-based event-pairing; however, a more reasonable distribution was obtained using
391 sequences inferred by continuous analysis, a method that is also parsimony-based but also

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392 incorporates branch length estimates. Thus, in our analyses, the latter method may be more
393 appropriate for estimating the ancestral sequences. The results also indicated that the potential
394 distribution of heterochronic shifts over the phylogenic tree is arbitrary and depends on the
395 optimization protocol. To assess the evolutionary distribution of heterochronic shifts, a more
396 effective high-resolution approach is needed. A potential alternative would be a
397 simulation-based approach combining a stochastic model with parsimony calculation. This
398 would require rigorous calculations and considerations but deserve a future challenge.

399 It is not clear whether teleost-specific circumstances are reflected in the present
400 results. For example, external temperature is known to affect developmental time frames
401 (Mabee, Olmstead & Cubbage, 2000; Schmidt & Starck, 2010) and, as most teleosts reproduce
402 by external fertilization and the embryos develop under fluctuating temperatures, temporal
403 shifts in individual developmental events may sometimes occur in teleosts in a natural
404 environment. Accordingly, teleosts may be naturally accustomed to sporadic shifts in
405 developmental events that may increase the probability of a shift being adopted in a persistent
406 manner. Future analyses using other groups of animals will address the specificity and
407 generality of the current results in evolutionary development.

408

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735 **Author contribution**

736 F.I. designed the study, performed the majority of the experiments, analyzed data and wrote the
737 manuscript. T.M contributed to designing and conducting the simulation. T.M. and T.H.
738 supervised the study and helped F.I. to write the manuscript with input from other people.

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741 **Competing interests**

742 The authors have no competing interests.

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745 **Figure Legends**

746 **FIGURE 1 Temporal orders of developmental events in the 31 actinopterygian species**

747 The temporal sequence of developmental events was extracted from the reference listed for each
748 species. Abbreviations of developmental events are: bc, blood circulation; cfr, caudal fin rays;
749 ep, eye pigmentation; es, embryonic shield; fs, first somite; h, hatching; hb, heart
750 beating/pulsing; kv, Kupffer's vesicle; le, lenses or lens placodes; mff, medial finfold; mo,
751 mouth opening; olf, olfactory vesicles/pits/placodes; oto, otoliths; ot, otic vesicles/
752 placodes/primordia; op, optic vesicles/placodes/primordia; pf, pectoral fin bud; tb, tail bud; tbr,
753 three brain regionalization; and tl, tail lift from the yolk. The ranks of missing data are marked
754 by ?.

755

756 **FIGURE 2 Phylogenetic relationships of the 31 actinopterygian species**

757 The phylogenetic tree of the 31 actinopterygian species examined in this study. The asterisk
758 marks anadromous teleosts, while all the others are freshwater teleosts. The numbers beside the
759 branches indicate the divergent times (Mya). The information sources for tree construction are
760 as follows: Near et al. (2012, 2013), Saitoh et al. (2011), Yang et al. (2015), Perez et al. (2007),
761 Friedman et al. (2013), Pohl, Milvertz, Meyer & Vences (2015).

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762

763 **FIGURE 3 Distribution of ranks of events in the developmental sequence**

764 The boxplot shows the statistical distribution (minimum, first quartile, median, third quartile,
765 maximum, and outliers) of normalized ranks for individual developmental events obtained from
766 the extant 30 teleost in-group data (a), and ancestral developmental sequences reconstructed by
767 acctran (b) and deltran (c) optimizations and continuous analysis (d). In all the panels, the
768 developmental events are aligned horizontally from left to right in the same order according to
769 the average ranks in the extant fish sequences.

770

771

772 **FIGURE 4 Rank changeability of individual developmental events during evolution**

773 (a) Rank variation is shown as the average of pairwise rank distances calculated by comparing
774 the pair of a sequence and its immediate ancestral sequence. The ancestral developmental
775 sequences were estimated using acctran (left) and deltran (right) optimizations of the
776 event-pairing method (Jeffery et al., 2002a). The events are vertically arranged from top to
777 bottom along the standard ontogenic time frame defined by the average developmental sequence
778 in the extant teleosts (Figure 2). (b) The evolutionary rate of rank changes for individual events

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779 obtained by continuous analysis (Germain & Laurin, 2009). The events are arranged in the same
780 order as in (a).
781
782 **FIGURE 5 Sequence orders of event pairs in extant developmental sequences**
783 The event sequence matrix represents all the pairwise combinations of developmental events.
784 The number shows the percentage of the sequences in which the row event occurred later than
785 the column event, and was calculated from the dataset of 30 extant teleost species, excluding the
786 missing event data. The individual cells are heatmap color-coded according to percentage value.
787
788 **FIGURE 6 Distribution of heterochronic shifts estimated by event-pairing in the teleost**
789 **phylogeny**
790 (a,b) Scatter plots showing the relationship between the phylogenetic branch length and number
791 of heterochronic shifts estimated from the extant and ancestral developmental sequences by
792 event-pairing with acctran (a) and deltran (b) optimizations (black circle). The reference
793 distribution (open circles) hypothesizes a linear correlation between the branch length and shift
794 number. (c) The coefficient of variation for the number of heterochronic shifts in a branch. The
795 black and open circles show the observed and reference values, respectively. The vertical bars

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796 indicate 95% confident intervals for the reference values. The branches are categorized into
797 three different groups: all branches, branches shorter than 50 million years (Mya), and branches
798 shorter than 20 Mya. (d) The number of branches without heterochronic shifts calculated from
799 observed (black circle) and reference (open circle) data in three different branch length
800 categories, as explained in (c). Vertical bars indicate 95% confident intervals of the reference
801 value. The number of branches in each analysis were 59 (all branches), 33 (shorter than 50
802 Mya), and 19 (shorter than 20 Mya).
803
804
805 **FIGURE 7 Comparison of the distribution of heterochronic rank changes estimated by**
806 **different methods**
807 (a to c) Scatter plots showing the relationship between branch length and the amount of total
808 rank changes estimated to have occurred in the branch. The ancestral developmental sequences
809 were estimated by event-pairing with acctran (a) and deltran (b) optimization, and continuous
810 analysis (c). (d) The number of total rank changes divided by the number of branches. The
811 coefficients of variation: 0.6405 (continuous), 0.6130 (acctran), and 0.8116 (deltran). (e) The
812 number of total rank changes per branch per million years. The coefficients of variation: 1.7396

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813 (continuous), 1.8769 (acctran), and 1.9670 (deltran).

Taxon	Common name	Reference	Ranks of events																		
			es	op	fs	tb	kv	tbr	ot	le	tl	hb	olf	mff	oto	bc	pfb	ep	h	mo	cfr
Amia calva	Bowfins	Ballard, 1986	1	2	2	2	?	3	4	5	5	5	5	6	8	7	7	7	10	8	9
Alosa sapidissima	American shad	Shardo, 1995	1	4	2	5	?	3	5	5	6	7	5	6	7	7	7	8	10	9	11
Catostomus commersoni	White sucker	Long and Ballard, 1976	1	3	2	3	4	3	4	5	5	6	7	6	7	7	8	8	8	10	9
Danio rerio	Zebrafish	Kimmel et al., 1995	1	4	3	2	4	5	5	6	5	8	7	7	7	9	9	8	10	11	11
Cyprinus carpio	Common carp	Verma, 1970	1	3	4	?	5	2	5	6	6	7	7	11	8	8	9	8	10	12	13
Carassius auratus	Minnows	Tsai et al., 2013	1	3	3	2	4	4	5	5	5	6	7	5	?	?	6	6	8	8	?
Barbodes goni[illegible]	Silver barb	[illegible]	1	3	2	2	?	[illegible]	7	4	5	6	?	6	?	5	6	[illegible]	9	10	8
Heteropneustes fossilis	Stinging catfish	Puvaneswari et al., 2009	1	2	2	2	4	3	6	4	4	5	5	4	?	7	9	8	7	10	8
Heterobranchus bidorsalis	African catfish	Olaniyi and Omitogun, 2014	1	2	2	2	3	7	4	8	4	5	8	?	4	8	?	5	6	9	10
Oncorhynchus mykiss	Rainbow trout	Ballard, 1973	1	3	2	?	2	3	7	5	6	6	4	7	9	7	7	8	12	10	11
Salmo salar	Atlantic salmon	Pelluet, 1944, Gorodilov, 1996	1	3	2	6	2	?	4	5	?	7	8	8	9	8	9	10	12	6	11
Galaxias maculatus	Common galaxias	Benzie, 1968	?	1	2	1	2	1	3	2	5	2	3	7	8	?	6	4	10	10	9
Gadus morhua	Atlantic cod	Hall et al., 2004	1	2	1	3	3	3	4	6	?	7	5	4	5	8	8	9	10	12	11
Gobius niger	Black goby	Ballard, 1969	1	2	2	3	3	6	5	4	4	8	?	5	7	9	8	8	10	11	12
Leucopsarion petersii	Ice goby	Arakawa et al., 1999	1	3	3	2	3	4	5	6	5	7	?	?	9	8	10	8	12	11	?
Gasterosteus aculeatus	Three-spined stickleback	Swarup, 1958	1	3	4	?	5	2	5	5	?	6	6	?	6	8	9	7	11	10	12
Stizostedion vitreum	Walleye	McElman and Balon, 1979	1	2	3	2	3	4	4	5	6	7	4	6	8	9	11	10	13	14	12
Channa striatus	Striped snakehead	Marimuthu and Haniffa, 2007	1	2	2	2	4	3	3	4	4	5	5	4	6	5	7	?	6	8	9
Anabas testudineus	Climbing gouramies	Zalina et al., 2012	1	3	4	?	2	4	4	5	?	6	?	?	7	7	?	10	8	9	11
Amphilophus xiloaensis	Cichlids	Kratochwil et al., 2015	1	2	2	2	?	5	2	4	3	5	?	?	?	7	8	8	6	9	10
Cichlasoma dimerus	South American cichlids	Meijide and Guerrero, 2000	1	4	3	2	?	5	5	5	6	5	?	8	6	6	9	8	7	10	10
Oreochromis niloticus	Nile tilapia	Fujimura and Okada, 2007	1	3	2	3	?	4	5	6	5	6	6	7	8	8	8	9	10	10	11
Labeotropheus trewavasae	Scrapermouth mbuna	Balon, 1977	1	2	4	3	?	2	3	5	5	6	11	9	5	7	9	8	10	12	13
Haplochromis piceatus	Victoria cichlids	de Jong et al., 2009	1	2	2	4	?	4	3	4	5	5	4	6	6	6	7	7	8	9	9
Melanotaenia splendida	Eastern rainbow fish	Humphrey et al., 2003	1	2	4	3	3	?	6	5	6	7	?	10	8	7	9	5	11	10	12
Adinia xenica	Diamond killfish	Cunningham and Balon, 1985	1	2	3	?	3	2	3	5	?	6	8	9	4	7	8	9	12	11	10
Fundulus heteroclitus	Mummichog	Armstrong and Swope Child, 1965	1	2	4	?	3	3	5	5	7	6	5	?	8	7	9	10	12	12	11
Xiphophorus maculatus	Southern platyfish	Tavolga and Rugh	1	2	2	4	?	3	3	5	4	5	6	?	8	6	5	7	11	10	9
Austrofundulus myersi	Rivulines	Wourms, 1998	1	4	3	2	2	5	6	7	10	7	?	11	9	8	9	10	14	13	12
Oryzias latipes	Japanese ricefish	Iwamatsu, 2004	1	3	4	?	2	5	4	6	9	7	13	12	8	8	10	11	16	14	15
Oryzias javanicus	Javanese ricefish	Iwamatsu and Hirata, 1984	1	3	4	?	2	5	5	6	9	7	7	?	8	8	9	10	13	12	11

Figure 1.

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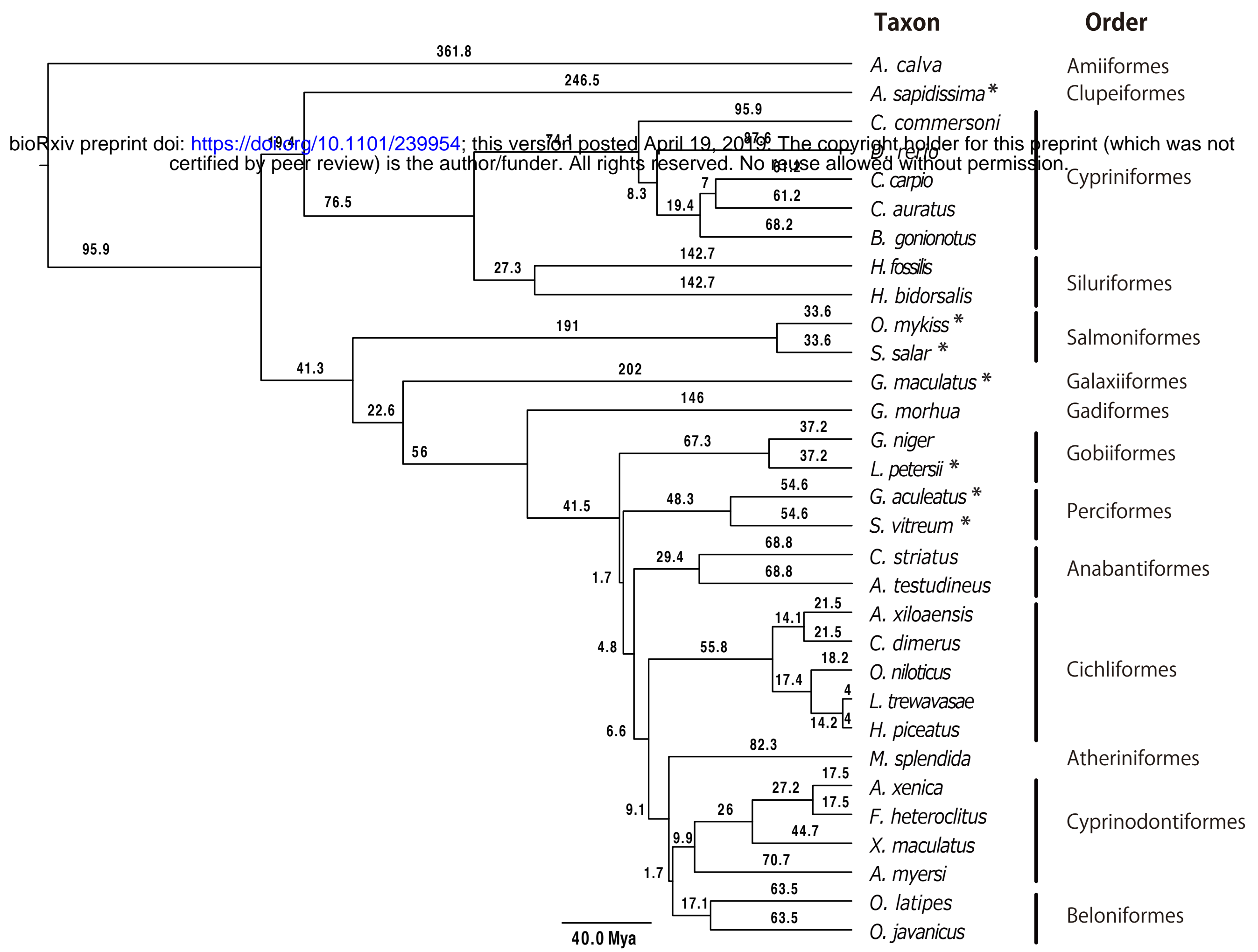
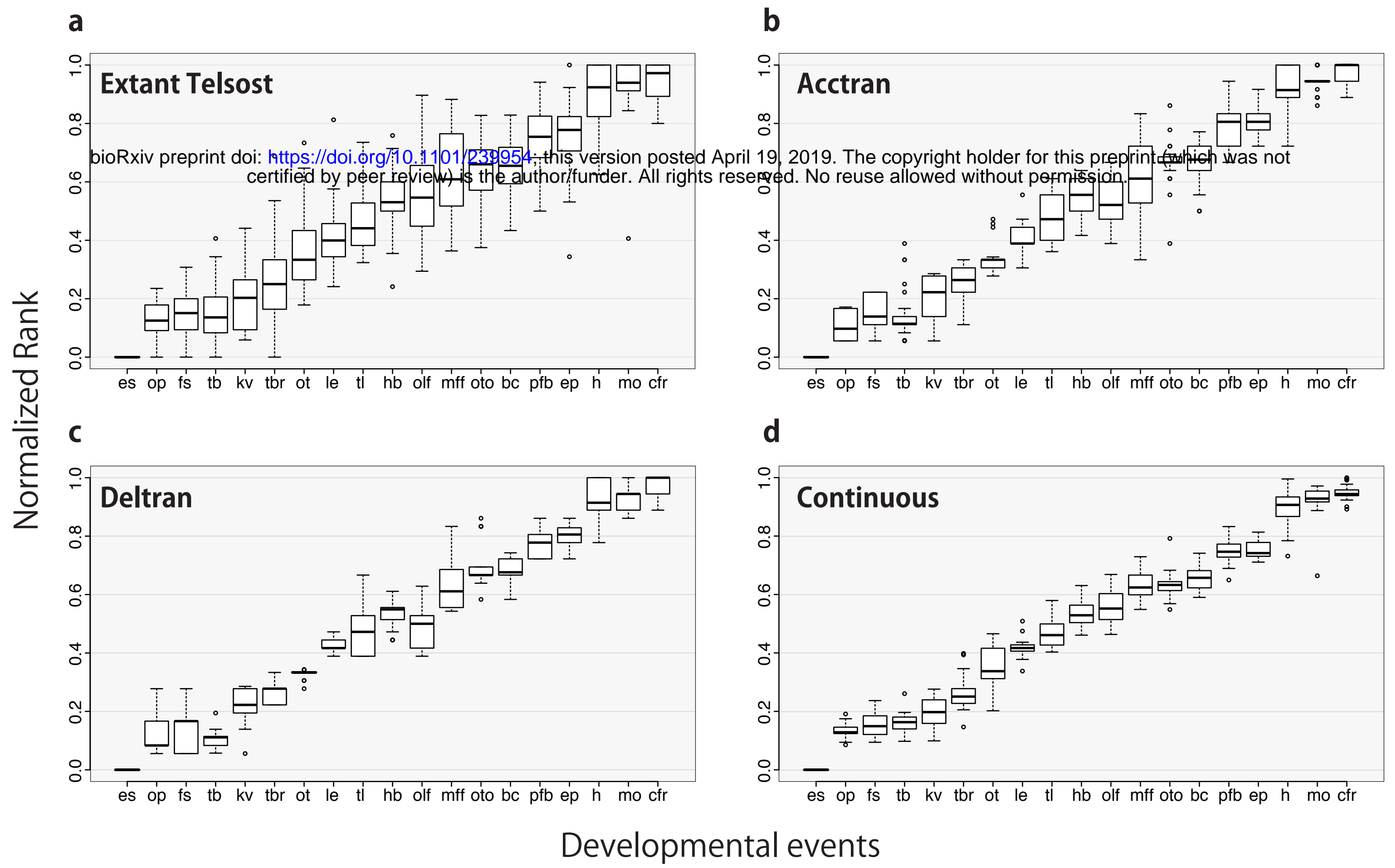


Figure 2.



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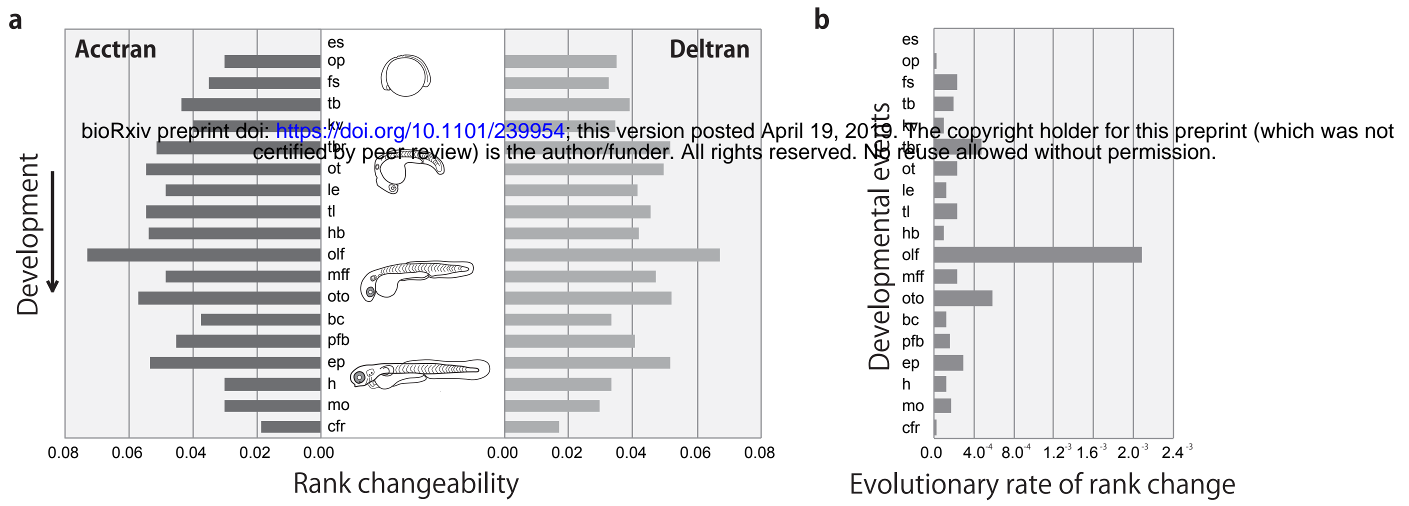

Figure 4

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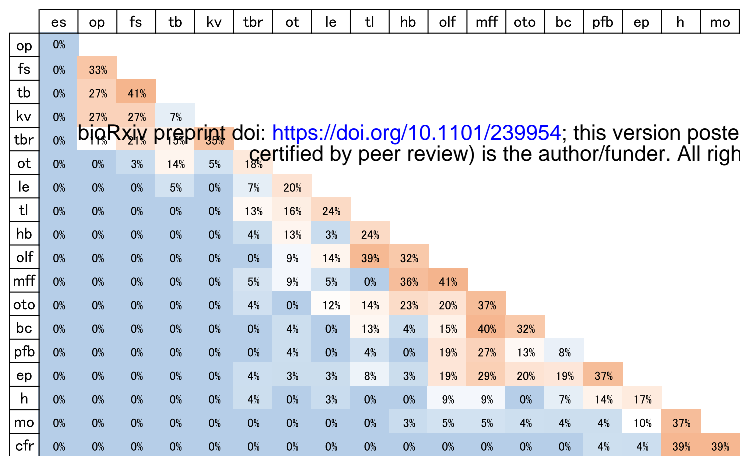

	es	op	fs	tb	kv	tbr	ot	le	tl	hb	olf	mff	oto	bc	pfb	ep	h	mo
op	0%																	
fs	0%	33%																
tb	0%	27%	41%															
kv	0%	27%	27%	7%														
tbr	0%	11%	21%	15%	35%													
ot	0%	0%	3%	14%	5%	18%												
le	0%	0%	0%	5%	0%	7%	20%											
tl	0%	0%	0%	0%	0%	13%	16%	24%										
hb	0%	0%	0%	0%	0%	4%	13%	3%	24%									
olf	0%	0%	0%	0%	0%	0%	9%	14%	39%	32%								
mff	0%	0%	0%	0%	0%	5%	9%	5%	0%	36%	41%							
oto	0%	0%	0%	0%	0%	4%	0%	12%	14%	23%	20%	37%						
bc	0%	0%	0%	0%	0%	0%	4%	0%	13%	4%	15%	40%	32%					
pfb	0%	0%	0%	0%	0%	0%	4%	0%	4%	0%	19%	27%	13%	8%				
ep	0%	0%	0%	0%	0%	4%	3%	3%	8%	3%	19%	29%	20%	19%	37%			
h	0%	0%	0%	0%	0%	4%	0%	3%	0%	0%	9%	9%	0%	7%	14%	17%		
mo	0%	0%	0%	0%	0%	0%	0%	0%	0%	3%	5%	5%	4%	4%	4%	10%	37%	
cfr	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	4%	4%	39%	39%

Figure 5.

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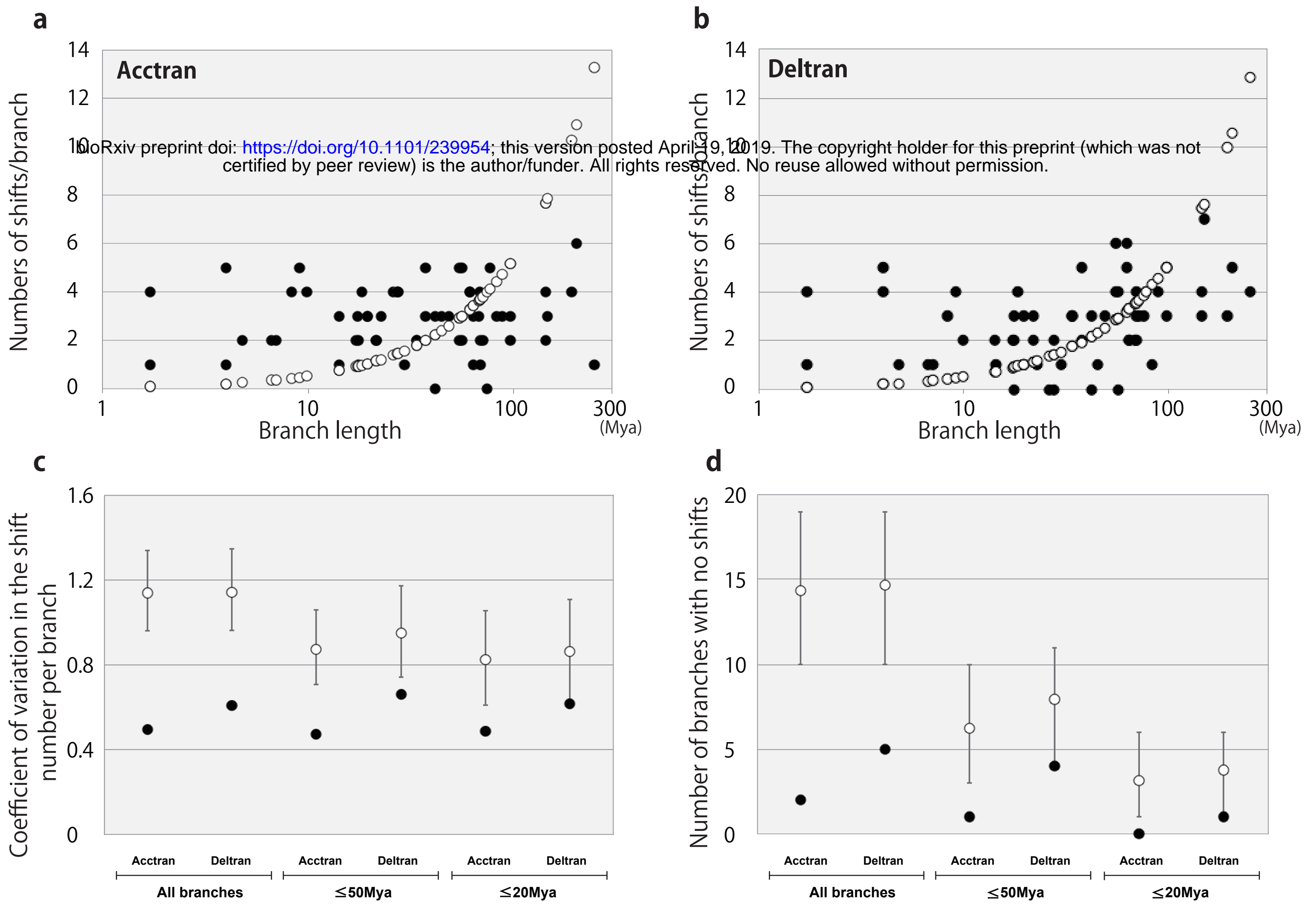

Figure 6.

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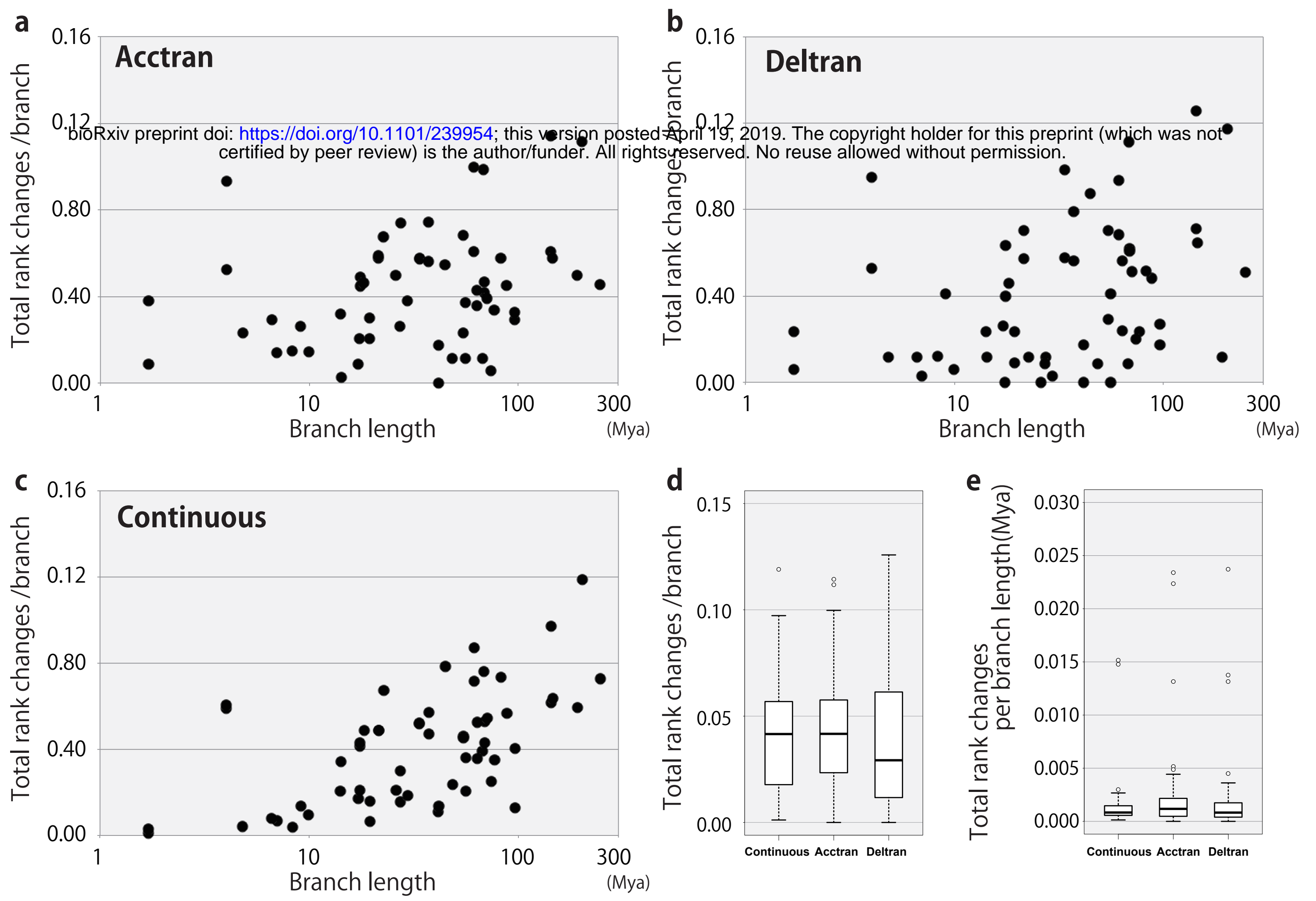

Figure 7.