Title: **Frequent Non-random Shifts in the Temporal Sequence of Developmental Landmark Events during Fish Evolutionary Diversification**

Running head: **Evolution of fish developmental sequence**

Authors

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Abstract

Morphology is a consequence of sequentially occurring developmental events, termed developmental sequence, and evolutionary changes in the sequence can generate morphological diversities. In this study, we examined evolutionary dynamics of the developmental sequence at a macro-evolutionary scale using the teleost fish. From the previous literature describing development of 31 fish species, we extracted the developmental sequences of 20 landmark events involving the whole body plan, and by using them, reconstructed ancestral developmental sequences. The phylogenetic comparisons of these sequences revealed event-dependent heterogeneity in the frequency of changing the sequences. We then determined potential event shifts that can parsimoniously explain the sequence changes on each node of the fish phylogenetic tree. These "heterochronic shifts" are widely distributed on almost of all the branches across the fish phylogeny. The simulation-based analysis indicated that this distribution of heterochronic shifts is not the result of random accumulation over phylogenetic time, but exhibits a curious constant trend so that individual phylogenetic branches harbor similar numbers of heterochronic shifts regardless of length. It is of great interest to know how these findings are related to morphological divergence in animals during evolution.

1. Introduction

The morphology of each multicellular organism is constructed by a fixed temporal sequence of

46 developmental events, termed developmental sequence. Because development is an inherently
47 step-by-step process, one might assume that the temporal sequence is not readily changeable
48 and is phylogenetically conserved among closely related species that share morphological
49 characteristics. Along these lines, if an evolutionary change occurs in the developmental
50 sequence, it could bring about a significant impact on animal body plan and lead to
51 morphological diversities. Indeed, previous comparisons of developmental sequences have
52 detected rare epoch-making changes that can provide morphological uniqueness to one species
53 that is different from the others (Strauss, 1990; Jeffery, Richardson, Coates & Bininda-Emonds,
54 2002; Maxwell, Harrison & Lasson, 2010) supporting the idea that the developmental sequence
55 is basically a conserved trait in the phylogenetic history.

56 Regarding evolution of the developmental sequences, another influential factor would
57 be the phylotypic period (Duboule, 1994). This is the developmental time frame during which
58 evolutionally distant animal species resemble each other in appearance. The well-accepted
59 hourglass-like model defines the phylotypic period as the middle phase of ontogenic
60 development, typically known as the pharyngulal stage. Recent transcriptome analyses have
61 indeed confirmed that inter-species diversities are kept to the minimum during this embryonic
62 stage (Kalinka et al., 2010; Irie & Kurtani, 2011), suggesting some unknown biological reasons
63 underlying this curious regularity. Although the phylotypic period was originally proposed by
64 the morphological resemblance, very few morphological analyses have actually been conducted
65 on species similarities during the period.

66 To explore the role for the developmental sequence in animal morphological evolution,
67 the critically missing information is empirical evaluation of evolutionary changes that had

actually occurred in the developmental sequences. In particular, very few systematic comparisons have been made on the sequences of a wide range of developmental events that cover the whole body plan in any class of animals. Therefore, we have few clues about how commonly or rarely the developmental sequences of these animals had changed during their evolutionary history. In the last several decades, comparative methods for developmental sequences have been developed by several groups (Nunn & Smith, 1998; Jeffery, Bininda-Emonds, Coates & Richardson, 2002; Jeffery, Bininda-Emonds, Coates & Richardson, 2005; Harrison & Larsson, 2008; Germain & Laurin, 2009). These methods compare the relative order of developmental events among different species and successfully detected potential evolutionary shifts of the events in a parsimonious manner, namely "heterochronic shifts" in developmental sequences (Schoch, 2006; Smirthwaitet, Rundle, Bininda-Emonds & Spicer, 2007; Sanchez-Villagra, Goswami, Weisbecker, Mock & Kuratani, 2008; Laurin, 2014; Carril & Tambussi, 2017). Although most of these analyses have so far focused on the developmental sequences for a particular organ or limited body part, we considered that the methods themselves are similarly applicable to a global analysis of developmental sequences for the whole body plan.

In this study, we conducted a comprehensive survey of developmental sequences using teleost fish. The teleost fish is the largest group of vertebrates. Its abundant group members are characterized by great morphological diversities (Nelson, Grande & Wilson, 2016) and, at the same time, share the common characteristics of the fish body plan such as vertebrae, eyes, medial fins and swim bladders (Romer & Parsons, 1986). Owing to the popularity as developmental research materials, there are well-established staging tables for many fish species

90 that cover common clear-cut developmental landmarks. Hence, the teleost fish can provide an
91 ideal dataset for systematic analyses of the early developmental sequences. Among the
92 widely-used developmental landmarks, we chose 20 events that individually contribute to
93 distinct body parts across the whole body plan. Using the dataset of 31 different fish species, we
94 compared the developmental sequences and reconstructed their ancestral sequences over the
95 phylogenetic tree. Our analysis indicated that the developmental sequences are in fact frequently
96 changeable during the course of evolution, and that these changes are associated with the two
97 following characteristics. (1) The frequency of sequence changes differs widely depending on
98 the developmental event. (2) Heterochronic shifts are not simply accumulated along the
99 phylogenetic time, but their number is kept more or less constant in individual phylogenetic
100 branches regardless of length. We will discuss the potential implications of these findings.
101

102 **2. Materials and Methods**

103 **2.1 Construction of fish phylogenetic tree**

104 The overall topology of the phylogenetic tree followed the molecular phylogenetic relationship
105 reported previously by Near et al. (2012, 2013). The minor branches missing in the tree were
106 inserted based on the phylogenetic data obtained from Saitoh et al. (2011) and Yang et al.
107 (2015) for Cypriniformes, Perez et al. (2007) and Friedman et al. (2013) for Cichliformes, and
108 Pohl, Milvertz, Meyer & Vences. (2015) for Cyprinodontiformes. The divergent times were
109 determined using the public database TIMETREE, *The Timescale of Life* (Hedes & Kumar,
110 2009) (Supplemental file.1).
111

112 **2.2 Data sampling**

113 Information about the temporal sequence of developmental events was extracted from 31
114 published research articles that describe normal fish development (Figure 1). The 20
115 developmental events used in this study were the first recognitions of blood circulation (bc),
116 caudal fin ray (cfr), eye pigmentation (ep), embryonic shield (es), first somite (fs), hatch (h),
117 heart beat/pulsing (hb), Kupffer's vesicle (kv), lens or lens placode/primodium (le), medial
118 finfold (mff), mouth opening (mo), olfactory vesicle/pit/placode (olf), otolithes (oto), otic
119 vesicle/placode/primodium (ot), optic vesicle/placode/primodium (op), pectoral fin bud (pfb),
120 swim bladder (sw), tail bud (tb), three brain regionalization (tbr), and tail lift from yolk (tl).
121 According to information in the articles, temporal orders of the developmental events were
122 ranked (Figure 1). When the article did not describe a developmental event, the event was
123 treated as a missing datum.

124

125 **2.3 Rank analyses of developmental events**

126 The raw ranks of individual developmental events were determined for the developmental
127 sequences of extant fish (Figure 1) and the ancestral developmental sequences reconstructed as
128 described below. The raw ranks were then normalized by the total number of the ranked events
129 (rmax) in each species, resulting in the relative scaling of the ranks in the range between $1/_{rmax}$
130 and 1 in all the species (Weisbecker, Goswami, Wroe & Sanchez-Villagra, 2008). To quantify
131 variation of the ranks among the developmental sequences, pairwise distances in the ancestral
132 ranks between all pairs of the sequences were summed and averaged for each pair of
133 combinations.

134

135 **2.4 Reconstruction of ancestral developmental sequences**

136 We used the event-pairing method developed by Jeffery et al. (2002). In brief, all of the 190
137 pairs of developmental events in each species were scored based on the relative timing; when
138 one event occurs earlier, simultaneously or later compared with another event, the timing was
139 rated as 0, 1, or 2, respectively. By comparing the scored event-pairing matrices of different
140 species, the ancestral event-pairing matrix was reconstructed at each node of the fish
141 phylogenetic tree in a parsimonious manner under accelerated transformation (acctran) and
142 delayed transformation (deltran) optimizations using PAUP* software (Swofford, 2002). The
143 ancestral event-pairing matrices were then re-converted to the ancestral developmental
144 sequences (Supplemental file.2).

145

146 **2.5 Detection of heterochronic shifts in fish phylogenetic tree**

147 The heterochronic shifts between two developmental sequences at each phylogenetic node were
148 detected using the Parsimov algorithm developed by Jeffery et al. (2005). This parsimony-based
149 algorithm determines the minimum number of event shifts that can explain the difference
150 between two developmental sequences. Following the instructions, we implemented a Perl
151 script, Parsimv7g.pl, with PAUP* output log file in both acctran and deltran optimizations, and
152 mapped the detected heterochronic shifts onto the fish phylogeny (Supplemental files 3).

153

154 **2.6 Simulation-base analyses**

155 We examined whether the estimated number of heterochronic shifts in each branch can be

simply explained by random accumulation in the phylogenetic tree. The simulation was based on a simple assumption that a heterochronic shift occurs at a constant rate per unit time and therefore, accumulates in proportion to branch length in the phylogenetic tree. In this simulation, we did not consider the event-dependent differences in the shift frequencies. The simulation randomly distributed the estimated heterochronic shifts over the fish phylogenic branches solely depending on their branch lengths. The simulation was replicated 100,000 times to obtain the expected distribution of heterochronic shifts in each branch under the assumption of random accumulation. The distribution of heterochronic shifts was then compared with the actual distribution of the hetrochronic shifts in the fish phylogenetic branches. In this study, we used year as the time scale of the branch length. However, in some analyses, we converted the time scale to generation by considering the average generation times of individual fish species (Supplemental file 4) and confirmed the consistency of the results.

3. Results

3.1 Phylogenetic relationship of 31 fish

For the present analyses, we used 30 teleost fish belonging to 13 distinct orders as the in-group, because the developmental sequences of these fish have been well-documented in previous articles (Figure 1). As an out-group, the amiadae fish, *Amia calva*, was used because it retains ancestral morphological characteristics and because a recent molecular analysis confirmed its location as the out-group of teleost fish (Near et al., 2012). In the constructed teleost phylogenetic tree, the examined fish species were widely distributed and represented distinct branches of teleost clade in a fairly unbiased manner (Figure 2). Because fish development in

178 the marine environment has rarely been documented, the fish species covered in this study were
179 basically freshwater fish, but also included several anadromous fish, such as *Gasterosteus*
180 *aculeatus*, which develop in freshwater but migrate between the sea and freshwater in their adult
181 life cycles.
182
183 **3.2 Comparison of temporal orders of developmental events among fish**
184 We selected 20 developmental events that consistently appeared as landmarks in the
185 developmental staging of many fish species (Figure 1). For this selection, in the hope to gain a
186 global picture of developmental sequences for the whole body plan, we intentionally included
187 events that belong to substantially different biological systems and contexts; e.g., the ones that
188 originate from different germ layers or that give rise to different cell types or separate body
189 parts. Additionally, the list also included a small number of seemingly interrelated events such
190 as formations of optic vesicle/placode/primodium (op), lens/lens placode (le) and eye
191 pigmentation (ep). We gathered information about these 20 events from the articles reporting
192 the development of 31 fish, and ranked the orders of individual events in the temporal sequence
193 for each species (Figure 1).
194 We first compared rank orders of each event among 30 in-group fish species. To
195 minimize effects of simultaneous occurrence of events and missing data on the comparison, the
196 raw ranks (Figure 1) were rescaled to normalized ranks that fit within the same range in all the
197 fish species (see Methods). Figure 3a shows distribution of the normalized ranks for individual
198 developmental events, which were horizontally arranged according to the average values.
199 Interestingly, the ranges of variations in the rank widely differed depending on the event. One

200 extreme case was embryonic shield (es), which always appeared first in the developmental
201 sequences obtained from the 29 fish species with no variation, excluding one missing
202 description in *Galaxias maculatus* (Figure 1). In contrast, relatively large variations in the rank
203 were observed for the appearance of Kupffer's vesicle (kv), hatch (h), medial finfold (mff) and
204 swim bladder (sb), suggesting that these events can more easily change their temporal orders in
205 the developmental sequence (Figure 3a).

206 To explore the evolutionary history of developmental sequences, we next
207 reconstructed ancestral developmental sequences at each node of the phylogenetic branches by
208 using the event-pairing method (Jeffery et al., 2002). This algorithm compares the relative
209 orders of all the event pairs between two different developmental sequences and generates the
210 ancestral sequences determined as a parsimonious solution under acctran and deltran
211 optimizations (Supplemental file 2). Using the obtained ancestral developmental sequences, we
212 compared the normalized ranks of individual events as we did in Figure 3a. Overall, the rank
213 orders of individual events in the ancestral developmental sequences (Figure 3b and 3c) were
214 quite similar to those in the extant fish sequences (Figure 3a); when the developmental events
215 were horizontally aligned in the same order of the extant average ranks, there were only a few
216 inversions in the order of two successive events at the average level (e.g., the order between first
217 somite (fs) and tail bud (tb)). Because this sequence reconstitution was based on parsimony, the
218 variations of estimated ranks were kept to nearly minimum. Still, individual events exhibited a
219 similar trend of rank variations to that observed in the extant fish sequences, further confirming
220 the idea that some developmental events change their orders more frequently than the others
221 during evolution.

222 Because the rank seemed to fluctuate depending on the event, we more
223 systematically analyzed the size of variations of the ranks. As an index of rank changeability,
224 the pairwise rank distances between all pairs of the ancestral developmental sequences were
225 measured and represented as the average value for each pair (Figure 4). Comparable values
226 were obtained by acctran and deltran optimizations (Spearman's rank correlation for the two
227 optimizations; r = 0.839). When the events were temporally arranged along the standard
228 ontogenic time frame defined as the average rank orders in the extant fish, the rank
229 changeability was found to be squeezed in the middle phase of the developmental sequence,
230 involving three brain regionalizations (tbr), otic vesicle/placode/primodium (ot) and lens or lens
231 placode/primodium (le) (Figure 4). The medial finfold (mff) around late-tail bud stage, in
232 contrast, recorded the largest rank changeability.

233 We then focused on the actual sequence of developmental events. Figure 5 shows the
234 percentage of the sequences in which one event (shown in row) occurs later than another
235 (shown in column) among the 30 extant in-group fish. In general, the sequence of two
236 temporally distant events was quite conservative, with no reversal in the order in many
237 combinations, whereas the neighboring events more frequently change their orders. If a closer
238 look was given to the sequence of anatomically interrelated events, the temporal order of the
239 optic vesicle/placode/primodium (op) and the lens/lens placode (le) was fixed in all the fish
240 species, and that of the lens/lens placode (le) and the eye pigmentation (ep) was almost fixed
241 except for one sequence reversal in *Heterobranchus bidorsalis*. Another interesting trend was
242 about the timing of hatch (h), which often changed the orders with the three late events, mouth
243 opening (mo), swim bladder (sb) and caudal fin ray (cfr). Similar results were obtained from the

244 comparison of event orders in ancestral developmental sequences (Figure S1).

245

246 **3.3 Distribution of heterochronic shifts across the fish phylogenetic tree**

247 Using the widely-used Parsimov algorithm (Jeffery et al., 2005), we next searched for
248 heterochronic shifts of the events that can explain the changes from one sequence to another at
249 every node of the fish phylogenetic tree. Although this is a parsimony-based algorithm and
250 therefore estimates the minimum number of event shifts, we detected 184 (acctran), 179
251 (deltran) and 94 (conserved between acctran and deltran) heterochronic shifts in total
252 (Supplemental file 3). The fish phylogenetic tree has 60 branches in total. When the detected
253 shifts were mapped over the phylogenetic tree, multiple heterochronic shifts were observed on
254 almost all the branches (Figure 2, Supplemental file 3). For example, in the relatively long
255 branch of order Salmoniformes, five and three heterochronic shifts were detected by acctran and
256 deltran optimizations, respectively.

257 Because a substantial number of heterochronic shifts were detected widely across the
258 fish phylogeny, we wondered whether these shifts might happen rather frequently and be
259 randomly accumulated over the evolutionary history. To address this question, we took a
260 simulation-based approach. Given that a heterochronic shift occurs at a random stochastic
261 manner and is neutrally accumulated, we simulated the expected distribution of the number of
262 heterochronic shifts, of which the number was nearly proportional to the phylogenetic branch
263 length (white circles in Figure 6a and 6b). By contrast, the actual distribution of heterochronic
264 shifts detected by the Parsimov analysis was much more constant, regardless of the branch
265 length in both acctran and deltran optimizations (black circles in Figure 6a and 6b). Coefficient

266 of variation of the number of heterochronic shifts across the branches also showed smaller
267 values for the experimental dataset than for the simulation data (Figure 6c), indicating that
268 branch-by-branch fluctuations of the number of heterochronic shifts are actually more limited
269 compared with the values expected under simulation. In addition, the number of the
270 phylogenetic branches that harbored no heterochronic shifts was significantly smaller for the
271 experimental dataset than that for the simulation data (Figure 6d). Because inclusion of an
272 extremely long branch could skew the statistical results, we performed the same statistical
273 comparison using only relatively short branches (≤ 50Mya and ≤ 20Mya). These analyses again
274 showed similar results, indicating that the number of heterochronic shifts per branch is more
275 constant than the expectation under the assumption of random accumulation (Figure 6c, 6d, and
276 Figure S2). Replacing the phylogenic time scale with the generation number basically did not
277 qualitatively affect the results of the analyses (Figures S3 and S4).

278 The heterochronic shifts of developmental events are sometimes associated to
279 differentiation of terminal phenotypes (Gunter, Koppermann & Meyer, 2014). Thus, we
280 examined the topological distribution of the heterochronic shifts by separately examining
281 internal and terminal branches. In both of the branch types, the numbers of actual heterochronic
282 shifts were basically in the range of the expected numbers in the simulation (Figure 6e and 6f).
283 Significant differences were only exceptionally observed in the all branch category under the
284 acctran optimization; however, we cannot rule out the possibility that the inclusion of extremely
285 long branches in this category affected the results. In conclusion, this analysis did not positively
286 support a preferential occurrence of heretochronic shifts in either the external or internal
287 branches.

4. Discussion

The present study provides empirical evidence that developmental sequences are in fact changeable during evolution; the extant fish species clearly involve historic signs showing that their ancestors had experienced dynamic and frequent rearrangement of the developmental sequences. This finding may not be exactly concordant with the traditional view that the developmental sequence is a phylogenetically conserved trait, which provides the blueprint for the common body plan among related species. One reason is probably our wide selection of developmental events; we intentionally took up the events that cover a whole variety of embryonic origins, cell types, body parts and biological systems, aiming for understanding the global body plan. In contrast, the major focus of previous studies was in-depth understanding of developmental sequences for a restricted body part or organ (Schlosser, 2008; Hautier et al., 2011; Workman, Charvet, Clancy, Darlington & Finlay, 2013). Therefore, even though we only analyzed one group of species that share the highly conserved body plan, rather frequent shifts of the events could be observed. There is increasing evidence for modular control of formation of different body parts (Klingenberg, Badyaev, Sowry & Beckwith 2001; Kawanishi et al., 2013; Schmidt & Starck, 2010). This modular nature of individual body parts can underlie the large fluctuations of developmental sequences observed in this study, and possibly contribute to individual evolution of different body parts toward morphological diversification.

Although the heterochronic shifts detected in this study are widespread across fish phylogeny, our simulation-based analyses uncovered certain regularity in the distribution. Namely, the shifts are not randomly accumulated over the evolutionary time, but there appears

310 to be some force making the number of shifts constant in individual phylogenetic branches.
311 Currently, we cannot effectively interpret this perplexing result, and only note two potential
312 scenarios from different angles, which are not necessarily mutually exclusive. First, it may be
313 possible that the heterochronic shift is a branching-related process. If a new branch often comes
314 with new shifts, then the shift number should be more correlated with the branch number rather
315 than its length. If speciation processes are concerned, this is actually a very attractive scenario,
316 because the heterochronic shift can support differentiation of branch-specific phenotypes in
317 each species. Second, the seeming constancy of the shift number might be related to the limited
318 configuration of acceptable developmental sequences. Our event sequence analyses indeed
319 showed that only certain types of changes are acceptable in the developmental sequences
320 (Figure 5). This limitation probably stems from both developmental and evolutionary
321 constraints in order to construct a fit functional body plan. Yet, for the moment, we cannot
322 determine how the limitation of sequence configurations can shape the distribution of potential
323 heterochronic shifts, because they are limited, but still a great many acceptable sequences exist.
324 One interesting finding of this study is that some developmental events change their
325 temporal orders more drastically than others during evolution. Of particular note is the
326 emergence of medial finfold (mff), of which rank changeability was the highest among all the
327 events (Figure 4). The medial finfold is a morphogenetic field for fins. A recent study reports
328 that this single morphogenetic field actually is a mosaic composed of three distinct fin primodia
329 (Larouch, Zelditch & Cloutier, 2017). Thus, it is possible that the three primodia behaved as
330 independent modules during evolution, thereby expanding the temporal range of this event.
331 Another interesting example is the timing of hatch (h), which is relatively easily changeable

332 with the three following developmental events, mouth opening (mo), swim bladder (sb) and
333 caudal fin ray (cfr). All these events are directly related to the life strategy of how a fish
334 survives during the larval stage, and therefore changing the orders may be advantageous under
335 some circumstances, particularly when fish have to adapt to a new environment (Miller &
336 Kendall, 2009). Indeed, the temporal shift of birth timing has been regarded as a symbolic
337 example of "heterochrony", an evolutionary force based on maneuvering developmental
338 machinery (Keyte & Smith, 2012; Dail, Reznick & Brainerd, 2017; Botelho, Smith-Paredes &
339 Vargas, 2015; Werneburg, Laurin, Koyabu & Sanchez-Villagra, 2016).

340 When the developmental events were aligned along the ontogenetic sequence, the rank
341 changeability was squeezed in the middle phase of the early development involving three brain
342 regionalization (tbr), otic placode/primodium (ot), and lens formation (le) (Figure 4). These
343 events are typical characteristics of the conserved phylotypic stage defined by the hourglass
344 model (Richardson, 1995; Irie, 2017). The hourglass model has been gaining increasing support
345 from the recent transcriptome analyses but still lacks sufficient evidence from objective
346 morphological analyses. Although the relationship between the developmental sequences and
347 morphological similarity is not very straightforward, our results seem to provide another
348 support for the hourglass model from the morphological point of view.

349 There is a common observation that the external temperature affects developmental
350 time frames (Schmidt & Starck, 2010; Mabee, Olmstead & Cubbage, 2000). Because most fish
351 reproduce by external fertilization and the embryos develop under fluctuating temperatures,
352 temporal shifts of individual developmental events might sometimes occur in fish under the
353 natural environment. Indeed, a study reports that the developmental sequence is polymorphic

354 even in one fish species (de Jong, Colbert, Witte & Richardson, 2009). Thus, fish may be
355 relatively tolerant to sporadic shifts of developmental events in the natural ontogeny, and
356 frequent encounters with such situations somehow increase the chances that some fish adopt a
357 shift in a persistent manner. Currently, it is not clear whether such fish-specific circumstances
358 are reflected in the present results. Future systematic analyses using other groups of animals
359 will address this issue.
360

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Author contribution

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

Competing interests

The authors have no competing interests.

Figure Legends

FIGURE 1 Temporal orders of developmental events in the 31 fish

The temporal sequence of developmental events was extracted from the reference listed for each species. Abbreviations of developmental events are; bc: blood circulation, cfr: caudal fin ray, ep: eye pigmentation, es: embryonic shield, fs: first somite, h: hatch, hb: heart beat/pulsing, kv: kupffer's vesicle, le: lens or lens placode/primodium, mff: medial finfold, mo: mouth opening, olf: olfactory vesicle/pit/placode, oto: otolithes, ot: otic vesicle placode/primodium, op: optic vesicle/placode/primodium, pfb: pectoral fin bud, sw: swim bladder, tb: tail bud, tbr: three brain regionalization, and tl: tail lift from yolk. The ranks of missing data are marked by ?.

FIGURE 2 Phylogenetic relationships of the 31 fish

671 The phylogenetic tree of the 31 fish examined in this study. The asterisk marks the anadromous
672 fish, while all the others are freshwater fish. The numbers aside the branches indicate the
673 divergent times (Mya). In some representative branches, the numbers of heterochronic shifts
674 detected under acctran and deltran optimizations are shown in boxes.
675
676 **FIGURE 3 Distribution of ranks of events in the developmental sequence**
677 The boxplot shows the statistical distribution (minimum, first quartile, median, third quartile,
678 maximum and outliers) of normalized ranks for individual developmental events obtained from
679 the extant in-group 30 fish data (a) and reconstructed ancestral developmental sequences by
680 acctran (b) and deltran (c) optimizations. In all of the panels, the developmental events are
681 horizontally aligned from left to right according to average ranks in the extant fish sequences. In
682 the ancestral sequences (b, c), the average sequence is reversed between first somite (fs) and tail
683 bud (tb), between heart beats (hb) and olfactory vesicle/pit/placode (olf), and between blood
684 circulation (bc) and otolithes (oto). An additional inversion is observed between swim bladder
685 (sb) and caudal fin rays (cfr) in the deltran optimization (c).
686
687 **FIGURE 4 Rank changeability of individual developmental events**

688 The variation of the ranks is shown as the average value of pairwise rank distances, which are

689 calculated from all the pairs of ancestral developmental sequences reconstructed under acctran

690 (left) and deltran (right) optimizations. The events are arranged along the standard ontogenic

691 time frame defined by the average developmental sequence in extant fish (Figure 2) from top to

692 bottom. *significant differences ($P<0.05$) by Mann-Whitney U-test when comparing the values

693 of Kupffer's vesicle (kv) and three brain regionalization (tbr) and those of lens formation (le)

694 and tail lift from yolk (tl).

695

696 **FIGURE 5 Sequence orders of event pairs in extant developmental sequences**

697 The event sequence matrix represents all the pairwise combinations of developmental events.

698 The number shows the percentage of the sequences in which the row event occurs later than the

699 column event, and was calculated from the dataset of extant 30 fish excluding the missing event

700 data. The individual cells are differently heatmap color-coded depending on the percentage.

701

702 **FIGURE 6 Distribution of heterochronic shifts in the fish phylogeny**

703 (a,b) The relationship between the phylogenetic branch length and the number of herterochronic

704 shifts detected from the extant and ancestral developmental sequences (black circle) and

705 theoretically estimated by simulation (open circle) under acctran (a) and deltran (b)
706 optimizations. In the simulation, the branch length and the number of shifts are highly
707 correlated (Spearman's rank correlation coefficients; 0.9995 (acctran), 0.9995 (deltran)). (c) The
708 coefficient of variance for the number of heterochronic shifts in each branch. The black and
709 open circles show the experimental and simulated values, respectively. The vertical bars
710 indicate 95% confident intervals for the simulated value. The analysis was conducted with three
711 different branch categories: all branches, and the branches shorter than 50 and 20 million years
712 (Mys). (d) The number of branches with no heterochronic shifts calculated from experimental
713 (black circle) and simulation data (open circle) in three different branch length categories.
714 Vertical bars indicate 95% confident intervals of the simulated value. (e, f) The number of
715 heterochronic shifts detected in the external (e) and internal (f) branches calculated from
716 experimental (black circle) and simulation data (open circle). The branches are categorized into
717 three groups according to their lengths. Vertical bars indicate 95% confident intervals of the
718 simulated value.
719
720

721 **FIGURE**

722

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	sb	cfr
Amia calva	Bowfins	Ballard, 1986	1	2	2	2	?	3	4	5	5	5	5	6	8	7	7	7	10	8	9	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 & Ballard, 1976	1	3	2	3	4	3	4	5	5	6	7	6	7	7	8	8	8	10	11	9
Danio rerio	Zebrafish	Kimmel et al., 1995	1	4	3	2	4	5	5	6	5	8	7	7	7	9	9	8	11	12	10	12
Cyprinus carpio	Common carp	Verma, 1970	1	3	4	?	5	2	5	6	6	7	7	11	8	8	9	8	10	13	12	14
Carassius auratus	Minnows	Tsai et al., 2013	1	3	3	2	4	4	5	5	5	6	7	5	?	?	6	6	8	8	9	?
Barbodes gonionotus	Silver barb	Basak et al., 2014	1	3	2	2	?	3	7	4	5	6	?	6	?	5	6	11	9	10	12	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 & Omitogue, 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	13	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	11
Gobius niger	Black goby	Ballard, 1969	1	2	2	3	3	6	5	4	4	8	?	5	7	9	8	8	10	11	9	12
Leucopsarion petersii	Ice goby	Arakawa et al., 1999	1	3	3	2	3	4	5	6	5	7	?	?	9	8	10	8	13	11	12	?
Gasterosteus aculeatus	Three-spined stickleback	Swarup, 1958	1	3	4	?	5	2	5	5	?	6	6	?	6	8	9	7	11	10	12	12
Stizostedion vitreum	Walleye	McElman & Balon, 1979	1	2	3	2	3	4	4	5	6	7	4	6	8	9	11	10	13	14	15	12
Channa striatus	Striped snakehead	Marimuthu & Haniffa, 2007	1	2	2	2	4	3	3	4	4	5	5	4	6	5	7	?	6	8	7	9
Anabas testudineus	Climbing gouramies	Zalina et al., 2012	1	3	4	?	2	4	4	5	?	6	?	?	7	7	?	10	8	9	9	11
Amphilophus xiloaensis	Cichlids	Kratochwil et al., 2015	1	2	2	2	?	5	2	4	3	5	?	?	?	7	8	8	6	9	11	10
Cichlasoma dimerus	South American cichlids	Meijide & Guerrero, 2000	1	4	3	2	?	5	5	5	6	5	?	8	6	6	9	8	7	10	?	10
Oreochromis niloticus	Nile tilapia	Fujimura & Okada, 2007	1	3	2	3	?	4	5	6	5	6	6	7	8	8	8	9	10	10	12	11
Labeotropheus trewavasae	Scrapermouth mbuna	Balon, 1977	1	2	4	3	?	2	3	5	5	6	11	9	5	7	9	8	10	12	14	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	6	9
Melanotaenia splendida	Eastern rainbow fish	Humphrey et al., 2003	1	2	4	3	3	?	6	5	6	7	?	11	8	7	9	5	12	11	10	13
Adinia xenica	Diamond killfish	Cunningham & Balon, 1985	1	2	3	?	3	2	3	5	?	6	8	9	4	7	8	9	13	12	11	10
Fundulus heteroclitus	Mummichog	Armstrong & Swope Child, 1965	1	2	4	?	3	3	5	5	7	6	5	?	8	7	9	10	12	12	12	11
Xiphophorus maculatus	Southern platyfish	Tavolga & 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	11	12
Oryzias latipes	Japanese ricefish	Iwamatsu & Hirata, 1984	1	3	4	?	2	5	4	6	9	7	13	12	8	8	10	11	17	15	14	16
Oryzias javanicus	Javanese ricefish	Iwamatsu, 2004	1	3	4	?	2	5	5	6	9	7	7	?	8	8	9	10	14	13	11	12

723 **FIGURE 1**

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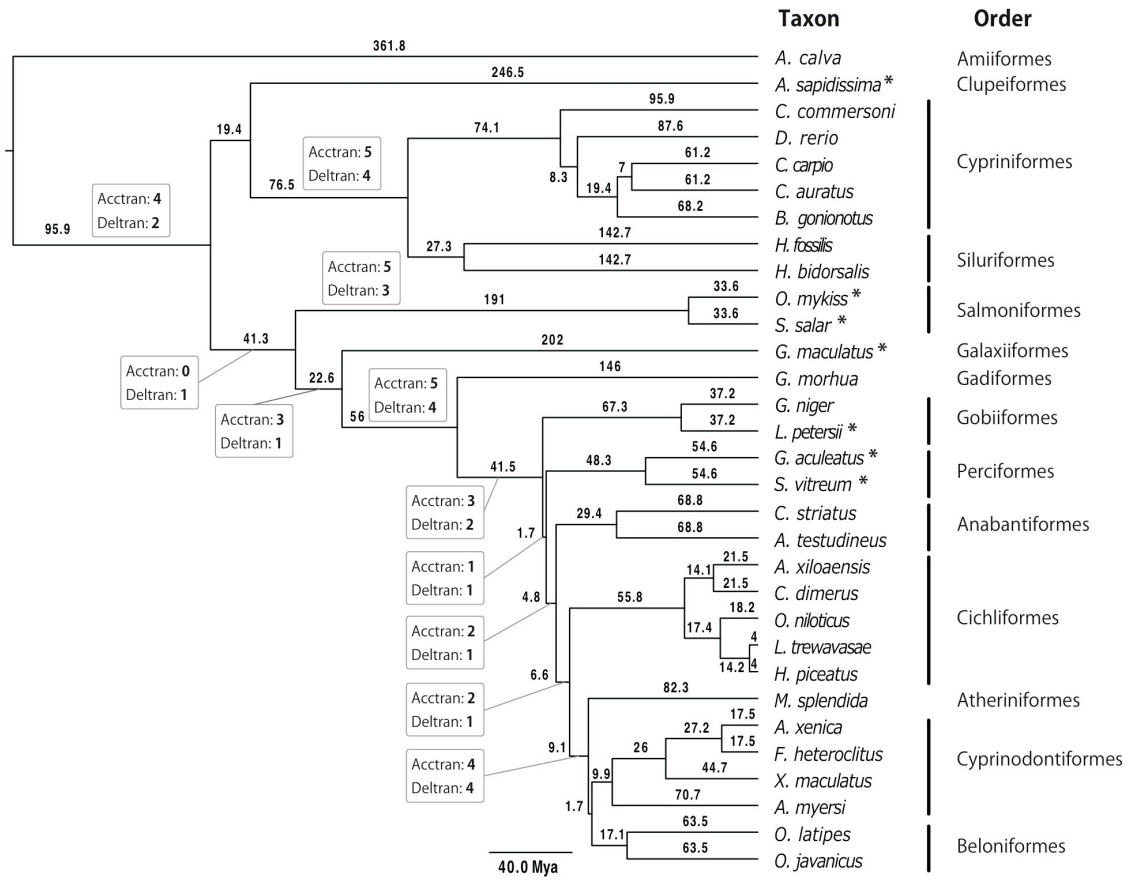
Taxon
Order
A. calva
A. sapidissima *
C. commersoni
D. rerio
C. carpio
C. auratus
B. gonionotus
H. fossilis
H. bidorsalis
O. mykiss *
S. salar *
G. maculatus *
G. morhua
G. niger
L. petersii *
G. aculeatus *
S. vitreum *
C. striatus
A. testudineus
A. xiloaensis
C. dimerus
O. niloticus
L. trewavasae
H. piceatus
M. splendida
A. xenica
F. heteroclitus
X. maculatus
A. myersi
O. latipes
O. javanicus
Amiiformes
Clupeiformes
Cypriniformes
Siluriformes
Salmoniformes
Galaxiiformes
Gadiformes
Gobiiformes
Perciformes
Anabantiformes
Cichliformes
Atheriniformes
Cyprinodontiformes
Beloniformes
Acctran: 4
Deltran: 2
Acctran: 5
Deltran: 4
Acctran: 5
Deltran: 3
Acctran: 0
Deltran: 1
Acctran: 3
Deltran: 1
Acctran: 5
Deltran: 4
Acctran: 3
Deltran: 2
Acctran: 1
Deltran: 1
Acctran: 2
Deltran: 1
Acctran: 2
Deltran: 1
Acctran: 4
Deltran: 4
40.0 Mya

FIGURE 2

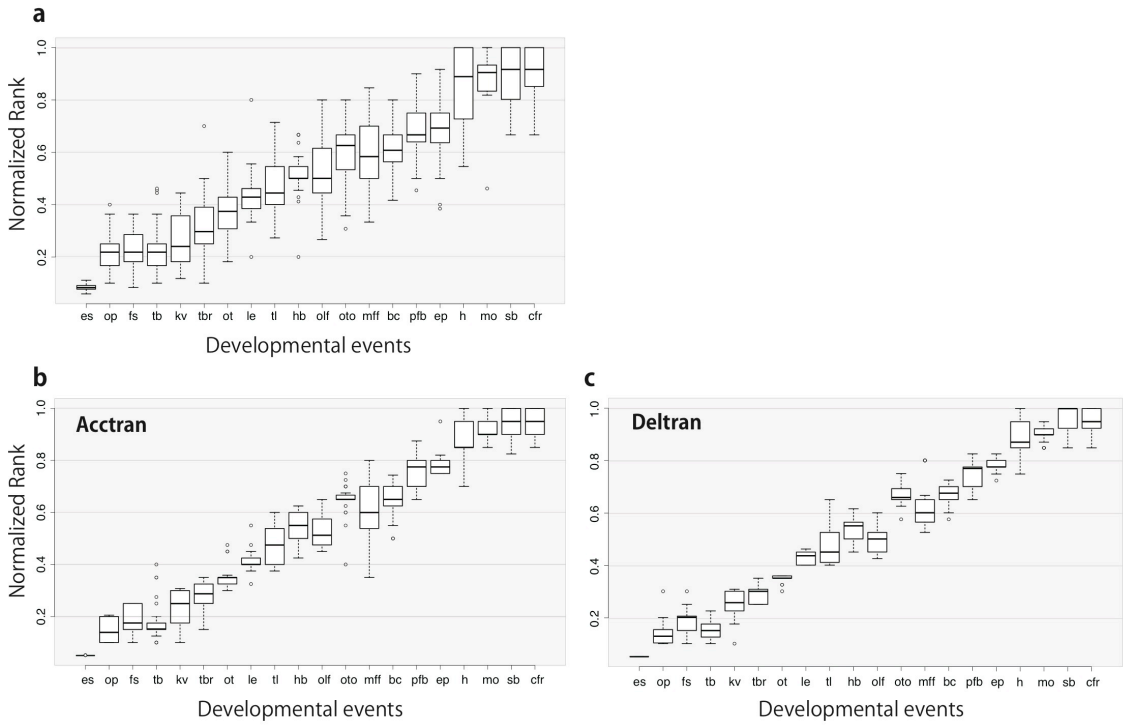
a
Normalized Rank
1.0
0.8
0.6
0.4
0.2
es op fs tb kv tbr ot le tl hb olf oto mff bc pfb ep h mo sb cfr
Developmental events
b
Acctran
Normalized Rank
1.0
0.8
0.6
0.4
0.2
es op fs tb kv tbr ot le tl hb olf oto mff bc pfb ep h mo sb cfr
Developmental events
c
Deltran
1.0
0.8
0.6
0.4
0.2
es op fs tb kv tbr ot le tl hb olf oto mff bc pfb ep h mo sb cfr
Developmental events

FIGURE 3

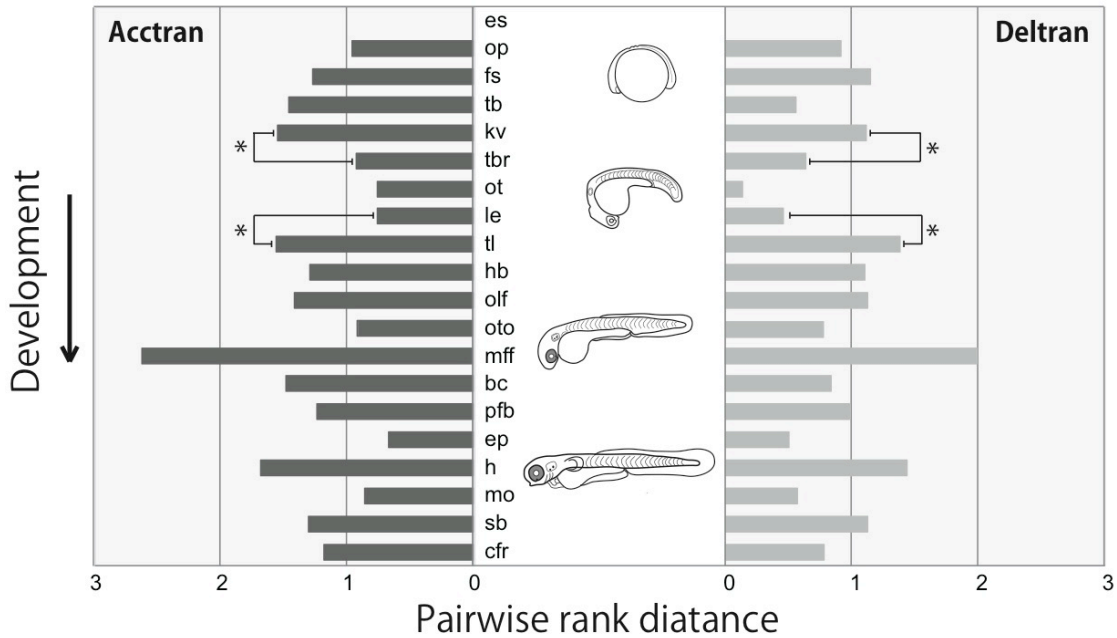
Acctran
Deltran
Development
es
op
fs
tb
kv
tbr
ot
le
tl
hb
olf
oto
mff
bc
pfb
ep
h
mo
sb
cfr
*
*
*
*
3
2
1
0
0
1
2
3
Pairwise rank diatance

FIGURE 4

	es	op	fs	tb	kv	tbr	ot	le	tl	hb	olf	oto	mff	bc	pfb	ep	h	mo	sb
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%									
oto	0%	0%	0%	0%	0%	5%	9%	5%	0%	36%	41%								
mff	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%		
sb	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	6%	0%	0%	5%	9%	39%	48%	
cfr	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	4%	4%	39%	39%	43%

FIGURE 5

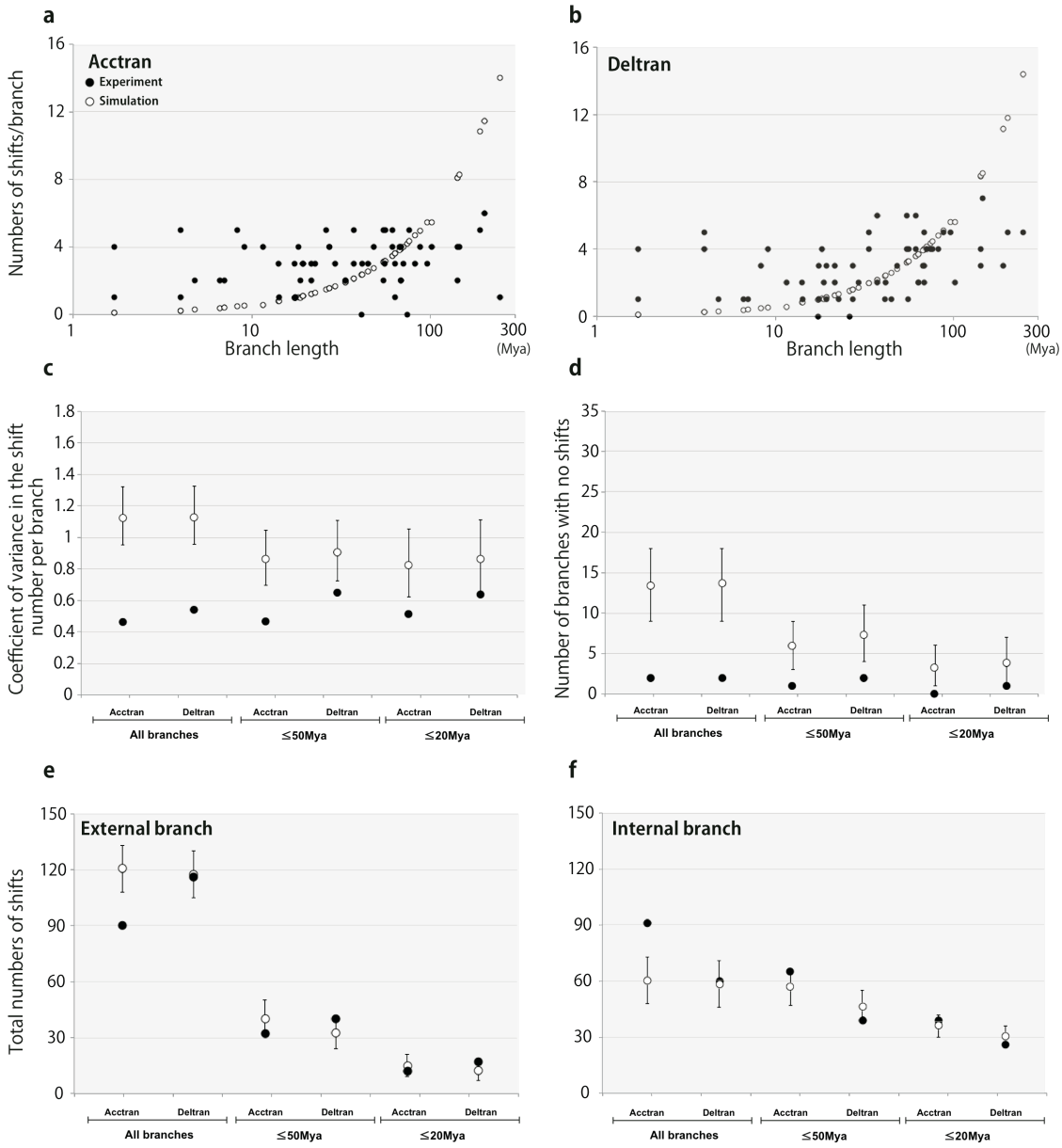
a
Acctran
Experiment
Simulation
Numbers of shifts/branch
Branch length
(Mya)
b
Deltran
Branch length
(Mya)
c
Coefficient of variance in the shift number per branch
d
Number of branches with no shifts
e
External branch
Total numbers of shifts
f
Internal branch
Acctran
Deltran
All branches
≤50Mya
≤20Mya

FIGURE 6