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

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Abstract

Morphology is a consequence of sequentially occurring developmental events, termed a 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.

Keywords

developmental sequence, evolution, heterochronic shifts, parsimov, teleost fish

1. Introduction

The morphology of each multicellular organism is constructed by a fixed temporal sequence of developmental events, termed developmental sequence. Because development is an inherently

step-by-step process, one might assume that the temporal sequence is not readily changeable and is phylogenetically conserved among closely related species that share morphological characteristics. Along these lines, if an evolutionary change occurs in the developmental sequence, it could bring about a significant impact on animal body plan and lead to morphological diversities. Indeed, previous comparisons of developmental sequences have detected rare epoch-making changes that can provide morphological uniqueness to one species that is different from the others [1 - 3] supporting the idea that the developmental sequence is basically a conserved trait in the phylogenetic history.

Regarding evolution of the developmental sequences, another influential factor would be the phylotypic period [4]. This is the developmental time frame during which evolutionally distant animal species resemble each other in appearance. The well-accepted hourglass-like model defines the phylotypic period as the middle phase of ontogenic development, typically known as the pharyngulal stage. Recent transcriptome analyses have indeed confirmed that inter-species diversities are kept to the minimum during this embryonic stage [5, 6], suggesting some unknown biological reasons underlying this curious regularity. Although the phylotypic period was originally proposed by the morphological resemblance, very few morphological analyses have actually been conducted on species similarities during the period.

To explore the role for the developmental sequence in animal morphological evolution, 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 [7 - 11]. 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 [12 – 16]. 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 [17] and, at the same time, share the common characteristics of the fish body plan such as vertebrae, eyes, medial fins and swim bladders [18]. Owing to the popularity as developmental research materials, there are well-established staging tables for many fish species that cover common clear-cut developmental landmarks. Hence, the teleost fish can provide an ideal dataset for systematic analyses of the early developmental sequences. Among the widely-used developmental landmarks, we chose 20 events that individually contribute to distinct body parts across the whole body plan. Using the dataset of 31 different fish species, we compared the developmental sequences and reconstructed their ancestral sequences over the phylogenetic tree. Our analysis indicated that the developmental sequences are in fact frequently changeable during the course of evolution, and that these changes are associated with the two following characteristics. (1) The frequency of sequence changes differs widely depending on the developmental event. (2) Heterochronic shifts are not simply

accumulated along the phylogenetic time, but their number is kept more or less constant in individual phylogenetic branches regardless of length. We will discuss the potential implications of these findings.

2. Materials and methods

(a) Construction of fish phylogenetic tree

The overall topology of the phylogenetic tree followed the molecular phylogenetic relationship reported previously by Near et al. (2012, 2013) [19, 20]. The minor branches missing in the tree were inserted based on the phylogenetic data obtained from Saitoh et al. (2011) [21] and Yang et al. (2015) [22] for Cypriniformes, Perez et al. (2007) [23] and Friedman et al. (2013) [24] for Cichliformes, and Pohl et al. (2015) [25] for Cyprinodontiformes. The divergent times were determined using the public database TIMETREE, *The Timescale of Life* [26] (Supplemental file.1).

(b) Data sampling

Information about the temporal sequence of developmental events was extracted from 31 published research articles that describe normal fish development (Figure 1) [27 - 57]. The 20 developmental events used in this study were the first recognitions of blood circulation (bc), caudal fin ray (cfr), eye pigmentation (ep), embryonic shield (es), first somite (fs), hatch (h), heart beat/pulsing (hb), Kupffer's vesicle (kv), lens or lens placode/primodium (le), medial finfold (mff), mouth opening (mo), olfactory vesicle/pit/placode (olf), otolithes (oto), otic vesicle/placode/primodium (ot), optic vesicle/placode/primodium (op), pectoral fin bud (pfb),

swim bladder (sw), tail bud (tb), three brain regionalization (tbr), and tail lift from yolk (tl). According to information in the articles, temporal orders of the developmental events were ranked (Figure 1). When the article did not describe a developmental event, the event was treated as a missing datum.

(c) Rank analyses of developmental events

The raw ranks of individual developmental events were determined for the developmental sequences of extant fish (Figure 1) and the ancestral developmental sequences reconstructed as described below. The raw ranks were then normalized by the total number of the ranked events (rmax) in each species, resulting in the relative scaling of the ranks in the range between $1/_{rmax}$ and 1 in all the species [58]. To quantify variation of the ranks among the developmental sequences, pairwise distances in the ancestral ranks between all pairs of the sequences were summed and averaged for each pair of combinations.

(d) Reconstruction of ancestral developmental sequences

We used the event-pairing method developed by Jeffery et al. (2002) [8]. In brief, all of the 190 pairs of developmental events in each species were scored based on the relative timing; when one event occurs earlier, simultaneously or later compared with another event, the timing was rated as 0, 1, or 2, respectively. By comparing the scored event-pairing matrices of different species, the ancestral event-pairing matrix was reconstructed at each node of the fish phylogenetic tree in a parsimonious manner under accelerated transformation (acctran) and delayed transformation (deltran) optimizations using PAUP* software [59]. The ancestral event-pairing matrices were

132 then re-converted to the ancestral developmental sequences (Supplemental file.2).

133

134 **(e) Detection of heterochronic shifts in fish phylogenetic tree**

135 The heterochronic shifts between two developmental sequences at each phylogenetic node were
136 detected using the Parsimov algorithm developed by Jeffery et al. (2005) [9]. This
137 parsimony-based algorithm determines the minimum number of event shifts that can explain the
138 difference between two developmental sequences. Following the instructions, we implemented a
139 Perl script, Parsimv7g.pl, with PAUP* output log file in both acctran and deltran optimizations,
140 and mapped the detected heterochronic shifts onto the fish phylogeny (Supplemental files 3).

141

142 **(f) Simulation-base analyses**

143 We examined whether the estimated number of heterochronic shifts in each branch can be simply
144 explained by random accumulation in the phylogenetic tree. The simulation was based on a
145 simple assumption that a heterochronic shift occurs at a constant rate per unit time and therefore,
146 accumulates in proportion to branch length in the phylogenetic tree. In this simulation, we did not
147 consider the event-dependent differences in the shift frequencies. The simulation randomly
148 distributed the estimated heterochronic shifts over the fish phylogenic branches solely depending
149 on their branch lengths. The simulation was replicated 100,000 times to obtain the expected
150 distribution of heterochronic shifts in each branch under the assumption of random accumulation.
151 The distribution of heterochronic shifts was then compared with the actual distribution of the
152 hetrochronic shifts in the fish phylogenetic branches. In this study, we used year as the time scale
153 of the branch length. However, in some analyses, we converted the time scale to generation by

154 considering the average generation times of individual fish species and confirmed the consistency
155 of the results (Supplemental file 4).
156
157 **3. Results**
158 **(a) Phylogenetic relationship of 31 fish**
159 For the present analyses, we used 30 teleost fish belonging to 13 distinct orders as the in-group,
160 because the developmental sequences of these fish have been well-documented in previous
161 articles (Figure 1). As an out-group, the amiadae fish, *Amia calva*, was used because it retains
162 ancestral morphological characteristics and because a recent molecular analysis confirmed its
163 location as the out-group of teleost fish [19]. In the constructed teleost phylogenetic tree, the
164 examined fish species were widely distributed and represented distinct branches of teleost clade
165 in a fairly unbiased manner (Figure 2). Because fish development in the marine environment has
166 rarely been documented, the fish species covered in this study were basically freshwater fish, but
167 also included several anadromous fish, such as *Gasterosteus aculeatus*, which develop in
168 freshwater but migrate between the sea and freshwater in their adult life cycles.
169
170 **(b) Comparison of temporal orders of developmental events among fish**
171 We selected 20 developmental events that consistently appeared as landmarks in the
172 developmental staging of many fish species (Figure 1). For this selection, in the hope to gain a
173 global picture of developmental sequences for the whole body plan, we intentionally included
174 events that belong to substantially different biological systems and contexts; e.g., the ones that
175 originate from different germ layers or that give rise to different cell types or separate body parts.

Additionally, the list also included a small number of seemingly interrelated events such as formations of optic vesicle/placode/primodium (op), lens/lens placode (le) and eye pigmentation (ep). We gathered information about these 20 events from the articles reporting the development of 31 fish, and ranked the orders of individual events in the temporal sequence for each species (Figure 1).

We first compared rank orders of each event among 30 in-group fish species. To minimize effects of simultaneous occurrence of events and missing data on the comparison, the raw ranks (Figure 1) were rescaled to normalized ranks that fit within the same range in all the fish species (see Methods). Figure 3a shows distribution of the normalized ranks for individual developmental events, which were horizontally arranged according to the average values. Interestingly, the ranges of variations in the rank widely differed depending on the event. One extreme case was embryonic shield (es), which always appeared first in the developmental sequences obtained from the 29 fish species with no variation, excluding one missing description in *Galaxias maculatus* (Figure 1). In contrast, relatively large variations in the rank were observed for the appearance of Kupffer's vesicle (kv), hatch (h), medial finfold (mff) and swim bladder (sb), suggesting that these events can more easily change their temporal orders in the developmental sequence (Figure 3a).

To explore the evolutionary history of developmental sequences, we next reconstructed ancestral developmental sequences at each node of the phylogenetic branches by using the event-pairing method [8]. This algorithm compares the relative orders of all the event pairs between two different developmental sequences and generates the ancestral sequences determined as a parsimonious solution under acctran and deltran optimizations (Supplemental file

198 2). Using the obtained ancestral developmental sequences, we compared the normalized ranks of
199 individual events as we did in Figure 3a. Overall, the rank orders of individual events in the
200 ancestral developmental sequences (Figure 3b and 3c) were quite similar to those in the extant
201 fish sequences (Figure 3a); when the developmental events were horizontally aligned in the same
202 order of the extant average ranks, there were only a few inversions in the order of two successive
203 events at the average level (e.g., the order between first somite (fs) and tail bud (tb)). Because this
204 sequence reconstitution was based on parsimony, the variations of estimated ranks were kept to
205 nearly minimum. Still, individual events exhibited a similar trend of rank variations to that
206 observed in the extant fish sequences, further confirming the idea that some developmental events
207 change their orders more frequently than the others during evolution.

208 Because the rank seemed to fluctuate depending on the event, we more systematically
209 analyzed the size of variations of the ranks. As an index of rank changeability, the pairwise rank
210 distances between all pairs of the ancestral developmental sequences were measured and
211 represented as the average value for each pair (Figure 4). Comparable values were obtained by
212 acctran and deltran optimizations (Spearman's rank correlation for the two optimizations; r =
213 0.839). When the events were temporally arranged along the standard ontogenic time frame
214 defined as the average rank orders in the extant fish, the rank changeability was found to be
215 squeezed in the middle phase of the developmental sequence, involving three brain
216 regionalizations (tbr), otic vesicle/placode/primodium (ot) and lens or lens placode/primodium
217 (le) (Figure 4). The medial finfold (mff) around late-tail bud stage, in contrast, recorded the
218 largest rank changeability.

219 We then focused on the actual sequence of developmental events. Figure 5 shows the

220 percentage of the sequences in which one event (shown in row) occurs later than another (shown
221 in column) among the 30 extant in-group fish. In general, the sequence of two temporally distant
222 events was quite conservative, with no reversal in the order in many combinations, whereas the
223 neighboring events more frequently change their orders. If a closer look was given to the
224 sequence of anatomically interrelated events, the temporal order of the optic
225 vesicle/placode/primodium (op) and the lens/lens placode (le) was fixed in all the fish species,
226 and that of the lens/lens placode (le) and the eye pigmentation (ep) was almost fixed except for
227 one sequence reversal in *Heterobranchus bidorsalis*. Another interesting trend was about the
228 timing of hatch (h), which often changed the orders with the three late events, mouth opening
229 (mo), swim bladder (sb) and caudal fin ray (cfr). Similar results were obtained from the
230 comparison of event orders in ancestral developmental sequences (Supplemental figure 1).
231
232 **(c) Distribution of heterochronic shifts across the fish phylogenetic tree**
233 Using the widely-used Parsimov algorithm [9], we next searched for heterochronic shifts of the
234 events that can explain the changes from one sequence to another at every node of the fish
235 phylogenetic tree. Although this is a parsimony-based algorithm and therefore estimates the
236 minimum number of event shifts, we detected 184 (acctran), 179 (deltran) and 94 (conserved
237 between acctran and deltran) heterochronic shifts in total (Supplemental file 3). The fish
238 phylogenetic tree has 60 branches in total. When the detected shifts were mapped over the
239 phylogenetic tree, multiple heterochronic shifts were observed on almost all the branches (Figure
240 2, Supplemental file 3). For example, in the relatively long branch of order Salmoniformes, five
241 and three heterochronic shifts were detected by acctran and deltran optimizations, respectively.

242 Because a substantial number of heterochronic shifts were detected widely across the
243 fish phylogeny, we wondered whether these shifts might happen rather frequently and be
244 randomly accumulated over the evolutionary history. To address this question, we took a
245 simulation-based approach. Given that a heterochronic shift occurs at a random stochastic
246 manner and is neutrally accumulated, we simulated the expected distribution of the number of
247 heterochronic shifts, of which the number was nearly proportional to the phylogenetic branch
248 length (white circles in Figure 6a and 6b). By contrast, the actual distribution of heterochronic
249 shifts detected by the Parsimov analysis was much more constant, regardless of the branch
250 length in both acctran and deltran optimizations (black circles in Figure 6a and 6b). Coefficient
251 of variation of the number of heterochronic shifts across the branches also showed smaller
252 values for the experimental dataset than for the simulation data (Figure 6c), indicating that
253 branch-by-branch fluctuations of the number of heterochronic shifts are actually more limited
254 compared with the values expected under simulation. In addition, the number of the
255 phylogenetic branches that harbored no heterochronic shifts was significantly smaller for the
256 experimental dataset than that for the simulation data (Figure 6d). Because inclusion of an
257 extremely long branch could skew the statistical results, we performed the same statistical
258 comparison using only relatively short branches ($\leq$ 50Mya and $\leq$ 20Mya). These analyses again
259 showed similar results, indicating that the number of heterochronic shifts per branch is more
260 constant than the expectation under the assumption of random accumulation (Figure 6c, 6d, and
261 Supplemental figure 2). Replacing the phylogenic time scale with the generation number
262 basically did not qualitatively affect the results of the analyses (Supplemental figures 3 and 4).
263 The heterochronic shifts of developmental events are sometimes associated to

differentiation of terminal phenotypes [60]. Thus, we examined the topological distribution of the heterochronic shifts by separately examining internal and terminal branches. In both of the branch types, the numbers of actual heterochronic shifts were basically in the range of the expected numbers in the simulation (Figure 6e and 6f). Significant differences were only exceptionally observed in the all branch category under the acctran optimization; however, we cannot rule out the possibility that the inclusion of extremely long branches in this category affected the results. In conclusion, this analysis did not positively support a preferential occurrence of heretochronic shifts in either the external or internal 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 [61 - 63]. 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 [64 - 66]. 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 to be some force making the number of shifts constant in individual phylogenetic branches. Currently, we cannot effectively interpret this perplexing result, and only note two potential scenarios from different angles, which are not necessarily mutually exclusive. First, it may be possible that the heterochronic shift is a branching-related process. If a new branch often comes with new shifts, then the shift number should be more correlated with the branch number rather than its length. If speciation processes are concerned, this is actually a very attractive scenario, because the heterochronic shift can support differentiation of branch-specific phenotypes in each species. Second, the seeming constancy of the shift number might be related to the limited configuration of acceptable developmental sequences. Our event sequence analyses indeed showed that only certain types of changes are acceptable in the developmental sequences (Figure 5). This limitation probably stems from both developmental and evolutionary constraints in order to construct a fit functional body plan. Yet, for the moment, we cannot determine how the limitation of sequence configurations can shape the distribution of potential heterochronic shifts, because they are limited, but still a great many acceptable sequences exist.

One interesting finding of this study is that some developmental events change their temporal orders more drastically than others during evolution. Of particular note is the

emergence of medial finfold (mff), of which rank changeability was the highest among all the events (Figure 4). The medial finfold is a morphogenetic field for fins. A recent study reports that this single morphogenetic field actually is a mosaic composed of three distinct fin primodia [67]. Thus, it is possible that the three primodia behaved as independent modules during evolution, thereby expanding the temporal range of this event. Another interesting example is the timing of hatch (h), which is relatively easily changeable with the three following developmental events, mouth opening (mo), swim bladder (sb) and caudal fin ray (cfr). All these events are directly related to the life strategy of how a fish survives during the larval stage, and therefore changing the orders may be advantageous under some circumstances, particularly when fish have to adapt to a new environment [68]. Indeed, the temporal shift of birth timing has been regarded as a symbolic example of "heterochrony", an evolutionary force based on maneuvering developmental machinery [69 - 72].

When the developmental events were aligned along the ontogenetic sequence, the rank changeability was squeezed in the middle phase of the early development involving three brain regionalization (tbr), otic placode/primodium (ot), and lens formation (le) (Figure 4). These events are typical characteristics of the conserved phylotypic stage defined by the hourglass model [73, 74]. The hourglass model has been gaining increasing support from the recent transcriptome analyses but still lacks sufficient evidence from objective morphological analyses. Although the relationship between the developmental sequences and morphological similarity is not very straightforward, our results seem to provide another support for the hourglass model from the morphological point of view.

There is a common observation that the external temperature affects developmental

time frames [66, 75]. Because most fish reproduce by external fertilization and the embryos develop under fluctuating temperatures, temporal shifts of individual developmental events might sometimes occur in fish under the natural environment. Indeed, a study reports that the developmental sequence is polymorphic even in one fish species [76]. Thus, fish may be relatively tolerant to sporadic shifts of developmental events in the natural ontogeny, and frequent encounters with such situations somehow increase the chances that some fish adopt a shift in a persistent manner. Currently, it is not clear whether such fish-specific circumstances are reflected in the present results. Future systematic analyses using other groups of animals will address this issue.

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631 **Author contribution**

632 F.I. designed the study, performed the majority of the experiments, analyzed data and wrote the
633 manuscript. T.M contributed to designing and conducting the simulation. T.M. and T.H.
634 supervised the study and helped F.I. to write the manuscript with input from other people.
635

636 **Competing interests**

637 The authors have no competing interests.
638

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643 parsimony analyses.

644 **Figure Legends**

645

646 **Figure 1. Temporal orders of developmental events in the 31 fish**

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

654

655 **Figure 2. Phylogenetic relationships of the 31 fish**

656 The phylogenetic tree of the 31 fish examined in this study. The asterisk marks the anadromous
657 fish, while all the others are freshwater fish. The numbers aside the branches indicate the
658 divergent times (Mya). In some representative branches, the numbers of heterochronic shifts
659 detected under acctran and deltran optimizations are shown in boxes.

660

661 **Figure 3. Distribution of ranks of events in the developmental sequence**

662 The boxplot shows the statistical distribution (minimum, first quartile, median, third quartile,
663 maximum and outliers) of normalized ranks for individual developmental events obtained from
664 the extant in-group 30 fish data (a) and reconstructed ancestral developmental sequences by
665 acctran (b) and deltran (c) optimizations. In all of the panels, the developmental events are

666 horizontally aligned from left to right according to average ranks in the extant fish sequences. In
667 the ancestral sequences (b, c), the average sequence is reversed between first somite (fs) and tail
668 bud (tb), between heart beats (hb) and olfactory vesicle/pit/placode (olf), and between blood
669 circulation (bc) and otolithes (oto). An additional inversion is observed between swim bladder
670 (sb) and caudal fin rays (cfr) in the deltran optimization (c).
671
672 **Figure 4. Rank changeability of individual developmental events**
673 The variation of the ranks is shown as the average value of pairwise rank distances, which are
674 calculated from all the pairs of ancestral developmental sequences reconstructed under acctran
675 (left) and deltran (right) optimizations. The events are arranged along the standard ontogenic
676 time frame defined by the average developmental sequence in extant fish (Figure 2) from top to
677 bottom. *significant differences ($P<0.05$) by Mann-Whitney U-test when comparing the values
678 of Kupffer's vesicle (kv) and three brain regionalization (tbr) and those of lens formation (le)
679 and tail lift from yolk (tl).
680
681 **Figure 5. Sequence orders of event pairs in extant developmental sequences**
682 The event sequence matrix represents all the pairwise combinations of developmental events.
683 The number shows the percentage of the sequences in which the row event occurs later than the
684 column event, and was calculated from the dataset of extant 30 fish excluding the missing event
685 data. The individual cells are differently heatmap color-coded depending on the percentage.
686
687 **Figure 6. Distribution of heterochronic shifts in the fish phylogeny**

688 (a,b) The relationship between the phylogenetic branch length and the number of herterochronic
689 shifts detected from the extant and ancestral developmental sequences (black circle) and
690 theoretically estimated by simulation (open circle) under acctran (a) and deltran (b)
691 optimizations. In the simulation, the branch length and the number of shifts are highly
692 correlated (Spearman's rank correlation coefficients; 0.9995 (acctran), 0.9995 (deltran)). (c) The
693 coefficient of variance for the number of heterochronic shifts in each branch. The black and
694 open circles show the experimental and simulated values, respectively. The vertical bars indicate
695 95% confident intervals for the simulated value. The analysis was conducted with three different
696 branch categories: all branches, and the branches shorter than 50 and 20 million years (Mys). (d)
697 The number of branches with no heterochronic shifts calculated from experimental (black
698 circle) and simulation data (open circle) in three different branch length categories. Vertical bars
699 indicate 95% confident intervals of the simulated value. (e, f) The number of heterochronic
700 shifts detected in the external (e) and internal (f) branches calculated from experimental (black
701 circle) and simulation data (open circle). The branches are categorized into three groups
702 according to their lengths. Vertical bars indicate 95% confident intervals of the simulated value.

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 [27]	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 [28]	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 [29]	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 [30]	1	4	3	2	4	5	5	6	5	8	7	7	7	9	9	8	11	12	10	12
Cyprinus carpio	Common carp	Vasma, 1970 [31]	1	3	4	2	5	2	5	6	6	7	7	11	8	9	9	8	10	13	12	14
Carassius auratus	Minnows	Asal et al., 2008 [32]	1	3	2	3	4	5	5	6	5	6	7	5	7	7	5	6	6	5	9	7
Barbodes gonionotus	Silver barb	Siddiqui et al., 2013 [33]	1	3	2	3	4	5	7	6	5	6	7	5	7	7	5	6	6	5	9	7
Heteropneustes fossilis	Stinging catfish	Puvaneswari et al, 2009 [34]	1	2	2	2	4	3	6	4	4	5	5	4	?	7	9	8	7	10	?	8
Heterobranchus bidorsalis	African catfish	Olaniyi and Omitogue, 2014 [35]	1	2	2	2	3	7	4	8	4	5	8	?	4	8	?	5	6	9	?	10
Oncorhynchus mykiss	Rainbow trout	Ballard, 1973 [36]	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 [37]	1	3	2	6	2	?	4	5	?	7	8	8	9	8	9	10	12	6	13	11
Galaxias maculatus	Common galaxias	Benzie, 1968 [38]	?	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 [39]	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 [40]	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 [41]	1	3	3	2	3	4	5	6	5	7	?	?	9	8	10	8	13	11	12	?
Gasterosteus aculeatus	Three-spined stickleback	Swarup, 1958 [42]	1	3	4	?	5	2	5	5	?	6	6	?	6	8	9	7	11	10	12	12
Stizostedion vitreum	Walleye	McElman and Balon, 1979 [43]	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 and Haniffa, 2007 [44]	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 [45]	1	3	4	?	2	4	4	5	?	6	?	?	7	7	?	10	8	9	9	11
Amphilophus xiloaensis	Cichlids	Kratochwil et al., 2015 [46]	1	2	2	2	?	5	2	4	3	5	?	?	?	7	8	8	6	9	11	10
Cichlasoma dimerus	South American cichlids	Meijide and Guerrero, 2000 [47]	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 [48]	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 [49]	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 [50]	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 [51]	1	2	4	3	3	?	6	5	6	7	?	11	8	7	9	5	12	11	10	13
Adinia xenica	Diamond killfish	Cunningham and Balon, 1985 [52]	1	2	3	?	3	2	3	5	?	6	8	9	4	7	8	9	13	12	11	10
Fundulus heteroclitus	Mummichog	Armstrong and Swope Child, 1965 [53]	1	2	4	?	3	3	5	5	7	6	5	?	8	7	9	10	12	12	12	11
Xiphophorus maculatus	Southern platyfish	Tavolga and Rugh [54]	1	2	2	4	?	3	3	5	4	5	6	?	8	6	5	7	11	10	?	9
Austrofundulus myersi	Rivulines	Wourms, 1998 [55]	1	4	3	2	2	5	6	7	10	7	?	11	9	8	9	10	14	13	11	12
Oryzias latipes	Japanese ricefish	Iwamatsu and Hirata, 1984 [56]	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 [57]	1	3	4	?	2	5	5	6	9	7	7	?	8	8	9	10	14	13	11	12

Figure 1.

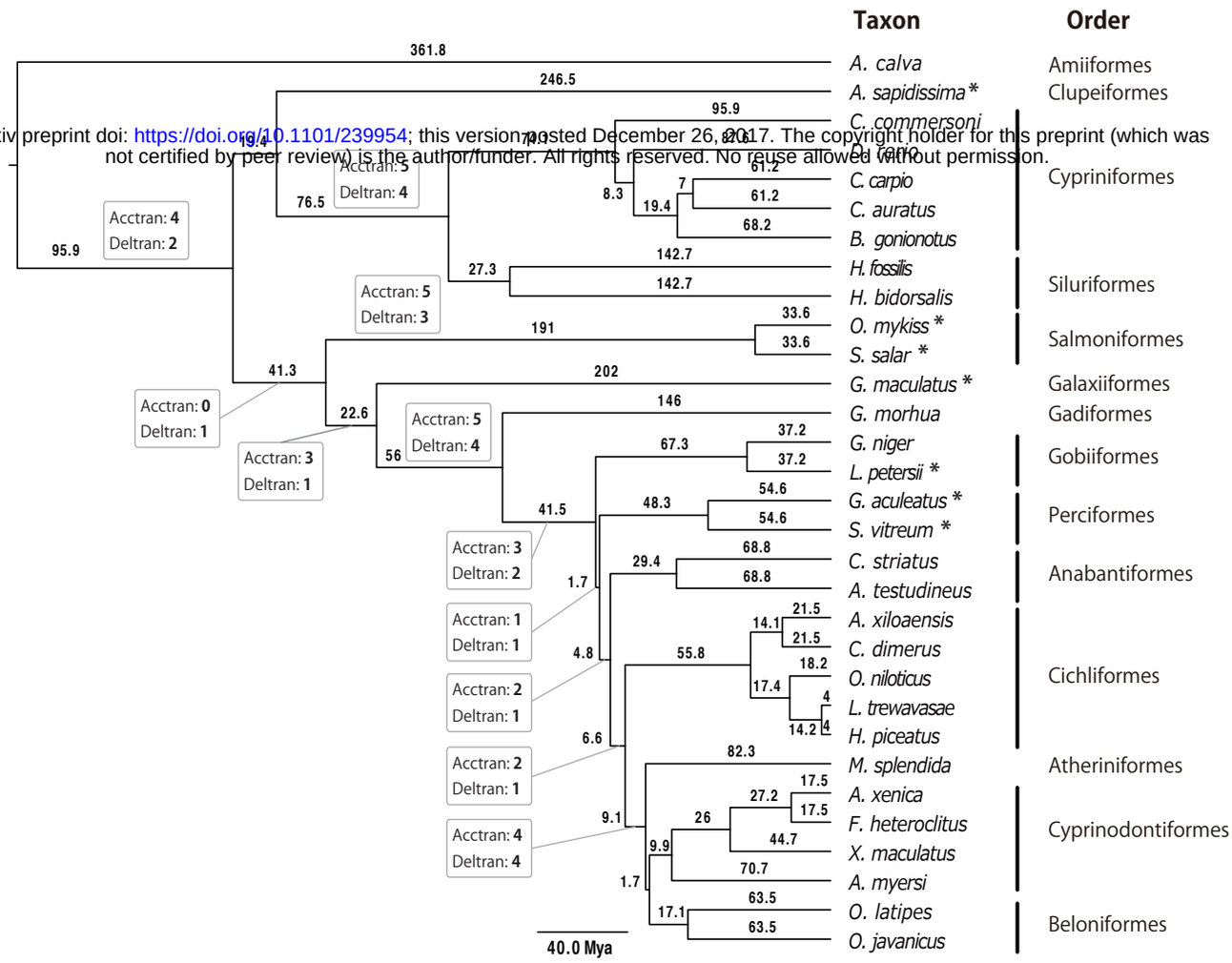
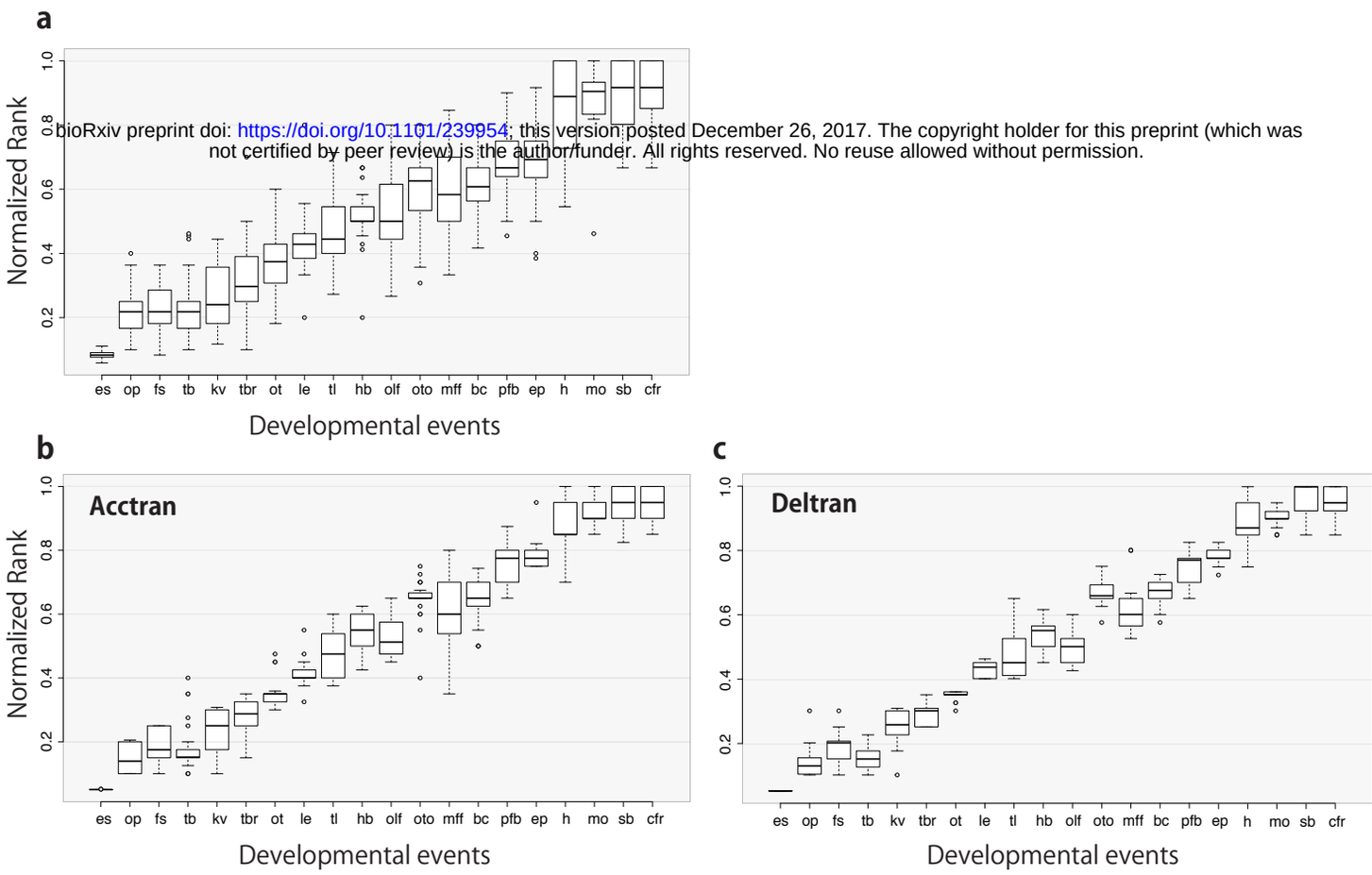


Figure 2.



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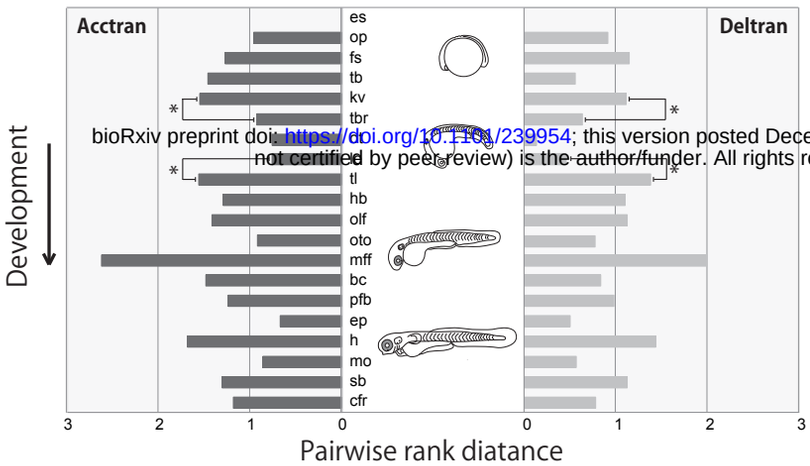

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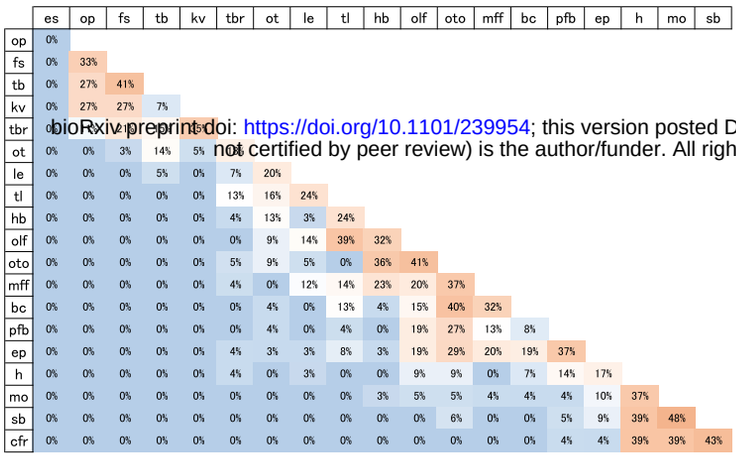

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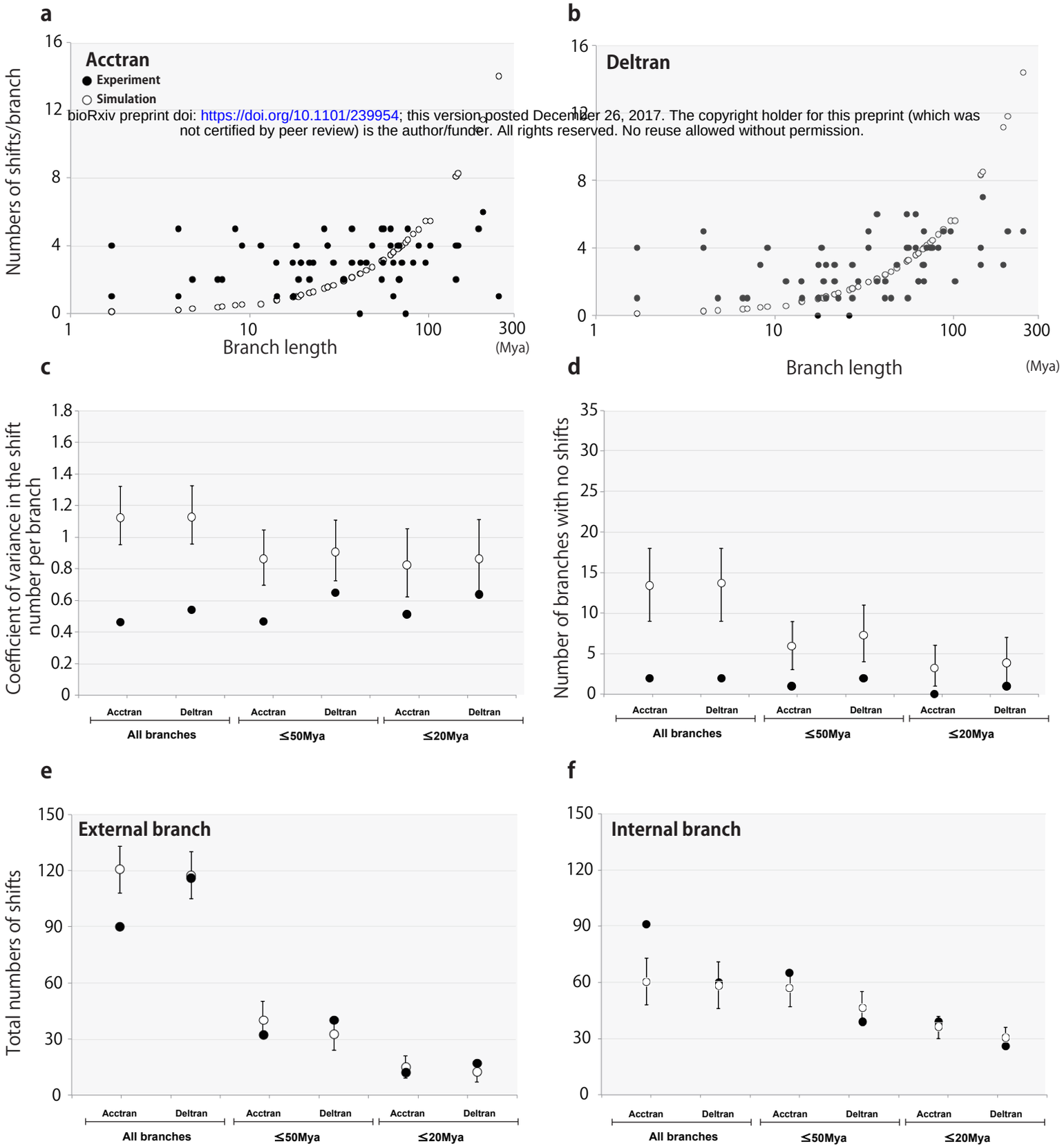

Figure 6.