Evolution of developmental sequence in teleost fish lineage

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CONTENTS

1.ABSTRACT

Morphology is a consequence of sequentially occurring of developmental events, termed developmental sequence, and evolutionary changes in the sequence can generate morphological diversities. Because in general, the evolutionary changes are recognized as a gradual process, it is assumed that developmental sequence also gradually evolved; closely related species would share both morphology and the sequence. However, so far, there are few pictures clearly showing their evolutionary relationships and underlying regulations. Hence, reconstructing the evolutionary history of developmental sequence would help to untangle progressions for morphological evolution. In this study, I examined evolutionary dynamics of the developmental sequence at a macro-evolutionary scale using teleost fish. From the previous literatures describing development of 31 fish species, I extracted 20 landmark developmental events that occurr sequentially in the whole fish body plan. First, I parsimoniously reconstructed the phylogenetic tree from the collected developmental sequence dataset. The topology of this tree was quite different from the molecular phylogenetic tree. This result implied that the developmental sequence of fishes has greatly rearranged during evolution, even between closely related species. Next, I reconstructed ancestral developmental sequences in fish molecular phylogenetic tree. The systematic comparisons of reconstructed ancestral sequences revealed that the frequent rearrangements of developmental sequences, and the frequency of sequence changes differed widely depending on individual developmental events. Then, by conducting two different methods, Parsimov and PGi, I detected potential event shifts that can parsimoniously

explain the sequence changes on each node of the fish phylogenetic tree. These "heterochronic shifts" were widely distributed on almost of all the branches across the fish phylogenetic tree. Then, collaborating with Dr. Tomotaka Matsumoto, we analyzed the distribution patterns of detected heterochronic shifts by a simulation-based approach. The simulation-based analysis indicated that the 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. Finally, I explored the relationship between developmental sequence and the duration of embryonic period. I reconstructed the evolutionary history of relative shifts of the hatch timing and the short duration of embryonic period, which revealed that these two changes seemed to be co-evolutionary phenomena; earlier shifts in the relative hatch timing accompanied shortening duration of embryonic period. This study provides an overview of evolution of developmental sequence in fish lineages by systematic analyses and discusses the underpinnings of morphological evolution.

2.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 diversity. 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 (Strauss 1990, Jeffery et al., 2002, Maxwell et al., 2010), supporting the idea that the developmental sequence is basically or partially a conserved trait in the phylogenetic history.

Regarding evolution of the developmental sequence, another influential factor would be the phylotypic period (Duboule 1994). 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 interspecies diversity is kept to the minimum during this embryonic stage (Kalinka et al., 2010, Irie and Kuratani 2011), suggesting some unknown biological reasons underlying this curious regularity. Detailed comparisons through morphogenesis are necessary to support the hourglass-like model. However, very few morphological analyses have actually been conducted on species similarities during the phylotypic period (Bininda-Emonds et al., 2003, Andrews et al., 2013).

In the ecological context, developmental sequence would be evolutionally optimized for post-embryonic environments for survival and reproductive strategies. So far, in mammal and bird clades, it was reported by several groups that changes of post-embryonic situations enhance developmental sequence rearrangements during embryonic period (Botelho et al., 2015, Werneburg et al., 2016). However, there were

few studies focusing on other animal groups, in which embryogenesis progress under various external environmental conditions. Furthermore, developmental sequence and duration of embryonic period were not well understood in fish clade.

To explore the role for the developmental sequence in animal morphological evolution, the critically missing information is empirical evaluation of evolutionary changes that 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 actually have few clues about how commonly or rarely the developmental sequences had changed during the evolutionary history. In the last several decades, comparative methods for the developmental sequences have been developed by several groups (Nunn and Smith 1998, Jeffery et al., 2002, Jeffery et al., 2005, Harrison and Larsson 2008, Germain and 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, that is "heterochronic shifts" in developmental sequences (Schoch 2006, Smirthwaite et al., 2007, Sanchez-Villagra et al., 2008, Laurin 2014, Carril and Tambussi, 2016). Although most of these analyses have so far focused on developmental sequences for a particular organ or a limited body part, the methods themselves are similarly applicable to a global analysis for the developmental sequence of the whole body plan.

In this study, I conducted a comprehensive survey of developmental sequences using teleost fish. Teleost fish is the largest group of vertebrates. Its group members are

characterized by great morphological diversities (Nelson et al., 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 and Parsons 1986). 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, I chose 20 events that individually contribute to distinct body parts across the whole body plan. Using the dataset of 31 different fish species, I compared the developmental sequences and reconstructed their ancestral sequences over the fish phylogenetic tree. These analyses indicated that the developmental sequences are in fact frequently changeable during the course of evolution, and that these changes are associated with the three following characteristics. (1) Heterochronic shifts frequently occurred over the fish phylogeny. (2) The frequency of sequence changes differs widely depending on the individual developmental events. (3) Based on simulation-based analyses, distribution of heterochronic shifts is not the result of the random accumulation over the phylogenetic time and similar numbers of heterochronic shifts occurred in individual branches regardless of their lengths. (4) The earlier shifts of relative hatch timing are co-evolved with the shortening duration of embryonic period.

3. MATERIALS AND METHODS

3.1.Construction of fish phylogenetic tree

The overall topology of the phylogenetic tree followed the molecular phylogenetic relationship reported previously by Near et al., 2012 and Near et al., 2013. The minor branches missing in the tree were inserted based on the phylogenetic data obtained from Saitoh et al., 2011 and Yang et al., 2015 for Cypriniformes, Perez et al., 2007 and Friedman et al., 2013 for Cichliformes, and Pohl et al., 2015 for Cyprinodontiformes (Figure 1). The divergent times were determined using the public database TIMETREE, *the Timescale of Life* (Hedges and Kumar 2009) (Table 1).

3.2. Data sampling

The information about the temporal sequence of developmental events was extracted from 31 published research articles that describe normal fish development (Table 2). 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 the description in the text and Figure legends of the articles, temporal orders of the developmental events were ranked (Table 2). When the article did not describe a developmental event, the event was treated as a missing datum.

3.3. Event-pairing matrix and reconstruction of the phylogenetic tree based on developmental sequence datasets

I used the event-pairing method developed by Jeffery et al., 2002 and scored all of the 190 event-pairs on event-pairing matrix, in each species by 0, 1 or 2 based on the relative timing of two developmental events; one event occurs earlier, simultaneously or later compared with another event, respectively. Then, I reconstructed parsimony tree using PAUP* software (Swofford, 2002) from the event-pairing matrix in heuristic search. Four parsimony trees were estimated in this reconstruction, and combined to a single consensus tree. The reconstructed tree length = 567.

3.4. Reconstruction of ancestral developmental sequences

By comparing the event-pairing matrices of different species, the ancestral event-pairing matrix was reconstructed at each node of the fish phylogenetic tree with parsimonious solution in both accelerated transformation (acctran) and delayed transformation (deltran) optimizations using PAUP* software (Swofford 2002). The reconstructed matrices at ancestral nodes were used for the reconstruction at further ancestral nodes. The ancestral sequence matrix was then re-converted to the ancestral developmental sequences (Table 3a, b).

3.5. Calculations of normalized rank and rank changeability

The raw ranks of individual developmental events were determined for the developmental sequences of extant fish (Table 2) 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 (Weisbecke et al, 2008). 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.

3.6. Detection of heterochronic shifts in fish phylogenetic tree by Parsimov method

The heterochronic shifts, which are relative event shifts between two developmental sequences at each phylogenetic node, were detected using the Parsimov algorithm developed by Jeffery et al., 2005. This parsimony-based algorithm determines the minimum number of event shifts that can explain the difference between two developmental sequences. Following the instructions, first I reconstructed ancestral event-pairing matrix by PAUP* in both acctran and deltran optimizations, and implemented a Perl script, *Parsimv7g.pl*, with the PAUP* output log file. The detected heterochronic shifts were mapped onto the fish phylogeny (Figure 8, Table 4).

3.7. Detection of heterochronic shifts in fish phylogenetic tree by PGi method

To detect heterocronic shifts by another algorithm, I used PGi (Parsimov-based genetic inference) method, which detects heterochronic shifts based on parsimoniously reconstructed ancestral developmental sequence (Harrison and Larsson 2008). I ran the PGi analysis four times independently and combined four obtained pseudoconsensus

trees with length of 223, 225, 221 and 216. Then, these preudoconsensus trees were combined to be a single superconsensus tree. The shifts with lower supporting values, which were calculated by bootstrap values, were cut off. The analytical parameters are follows: 100 cycles of selection per node, 100 sequences per cycle of selection, and a maximum of 100 ancestral developmental sequences to be retained at each node. The detected heterochronic shifts were mapped onto the fish phylogeny (Figure 9).

3.8. Simulation-based analyses

Collaborating with Dr. Tomotaka Matsumoto, 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 and

confirmed the consistency of the results (Table 5).

3.9. Inferring ancestral state by Mesquite reconstruction

To estimate the relationship between evolutionary shifts in relative hatch timing and shortening duration of embryonic period, I conducted Mesquite software (Maddison and Maddison 2015). First, I categorized species based on the relative hatch timing. The criteria are whether three or more events occurred after the hatch in the developmental sequence, or whether the duration from fertilization to hatch is shorter than 100 hours. Then, these information and phylogenetic topology were put into Mesquite software to parsimoniously reconstruct the ancestral state in each branch.

4. RESULTS

4.1. Phylogenetic of relationship of 31 fishes examined

For the present analyses, I used 30 teleost fishes belonging to 13 distinct orders as the in-group, because the developmental sequences of these fishes have been well documented in previous articles (Table 2). 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 fishes (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 1). Because fish development in the marine environment has rarely been documented, the fish species covered in this study were basically fresh water fish, but

also included several anadromous fishes, such as three-spined stickleback, which develop in fresh-water but migrate between the sea and fresh water in their adult life cycles.

4.2. Phylogenetic reconstruction by developmental sequence

I selected 20 developmental events that consistently appear as landmarks in the developmental staging of many fish species (Table 2). For this selection, in the hope to gain a global picture of developmental sequences for the whole body plan, I included events that belong to substantially different biological systems and contexts; e.g., the ones that originate from different germ layers, that give rise to different cell types and 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). I 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 (Table 2).

First, to check whether the closely related species shared similar developmental sequences, I reconstructed a parsimony tree from the event-pairing matrix. If the developmental sequences are similar among closely related species, the topology of this tree will be expected to be similar to that of molecular phylogeny. The reconstructed parsimony tree is shown in Figure 2. Only two species pairs in Beloniformes (*O.javanicus* and *O. latipes*) and Cichliformes (*A. xiloaensis* and *C. dimerus*) were closely located as the neighborings in both reconsrtucted tree by

event-pairing matrix and molecular phylogenetic trees. In the other cases, the topology was different from the molecular phylogenetic tree. Because the temporal order of developmental events did not accurately reconstruct the phylogenetic relationship, developmental sequences of teleost fish seem to diverge in different rate or process from the mutation accumulations in their genomes.

4.3. Comparison of temporal orders of developmental events among fishes

Next, I 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 (Table 2) were rescaled to normalized ranks that fit within the same range in all the fish species (see the Methods). Figure 3 shows distribution of the normalized ranks for individual developmental events, which are horizontally arranged according to the average rank 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 (Figure 3), except for one missing description in *Galaxias maculatus*. 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 3).

To explore the evolutionary history of developmental sequences, I next reconstructed ancestral developmental sequences at each node of the phylogenetic tree

by using the event-pairing method (Jeffery et al., 2002). 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 (Table 3a, b). Using the obtained ancestral developmental sequences, I compared the normalized ranks of individual events as shown in Figure 3. Overall, the rank orders of individual events in the ancestral developmental sequences (Figure 4a, b) were quite similar to those in the extant fish sequences (Figure 3); there were only a few inversions in the order of two successive events at the average level (e.g. the order between first somite (fs) and tail bud (tb)). The range of normalized rank variation in extant species were relatively larger than the ancestral range of variation. One potential reason is that the total number of the ranked events was different and smaller in extant fishes, and hence the effects of single rank change would be larger. Another potential reason is that, since acctran and deltran optimization have a tendency to estimate more shifts in internal and external branches, respectively, rank variation of acctran reconstruction can be larger than deltran.

Because this sequence reconstitution was based on parsimony, the variations of estimated ranks were kept to nearly minimum. Still, individual events exhibited a similar trend of rank variations to that observed in the extant fish sequences, further confirming the idea that some developmental events change their orders more frequently than the others during evolution.

4.4. Evolutionary rank changeability in ontogenetic context

Because the rank seemed to fluctuate depending on events, I more systematically analyzed the size of variations of the ranks. As an index of rank changeability, the pairwise rank distances between all pairs of the ancestral developmental sequences were measured and represented as the average value for each pair (Figure 5). Comparable values were obtained by acctran and deltran optimizations (Spearman's rank correlation for the two optimizations; $r = 0.839$). When the events were arranged along the standard ontogenic time frame defined as the average rank orders in the extant fishes, the rank changeability was found to be squeezed in the middle phase of developmental sequence, involving three brains regionalizations (tbr), otic vesicle/placode/primodium (ot) and lens or lens placode/primodium (le) (Figure 5). The medial finfold (mff) around late-tail bud stage, in contrast, recorded the largest rank changeability.

4.5. Frequency of sequence reversion among extant fishes

I then focused on the actual sequence of developmental events. Figure 6 shows the percentage of the sequences in which one event (shown in row) occurs later than another (shown in column) among the 30 extant in-group fishes. In general, the sequence of two temporally distant events was quite conservative with no reversal in the order in many combinations, whereas the neighboring events more frequently change their orders. If a closer look was given to the sequence of anatomically interrelated events, the temporal order of the optic vesicle/placode/primodium (op) and the lens/lens placode (le) was fixed in all the fish species, and that of the lens/lens placode (le) and the eye pigmentation (ep) was almost fixed except for one sequence reversal in

Heterobranchus bidorsalis. Another interesting trend was about the timing of hatch (h), which often changed the orders with the three late events, mouth opening (mo), swim bladder (sb) and caudal fin ray (cfr). Similar results were obtained from the comparison of event orders in ancestral developmental sequences (Figure 7).

4.6. Detection of heterochronic shifts across the fish phylogenetic tree by Parsimov and PGi methods

Using the widely-used Parsimov algorithm (Jeffery et al., 2005), I searched for heterochronic shifts of the events that can explain the changes from one sequence to another at every node of the fish phylogenetic tree. This was a parsimony-based algorithm and therefore should estimate the minimum number of event sifts but I detected 184 (acctran), 179 (deltran) and 94 (conserved between acctran and deltran) heterochronic shifts in total (Figure 8, Table 4). When the detected shifts were mapped over the phylogenetic tree, heterochronic shifts were observed on all the branches (Figure 8, Table 4).

Next, to confirm whether the other algorithm led to similar results, I performed PGi analysis, which was another widely-used method for detecting heterochronic shifts (Harrison and Larsson 2008). The PGi, which is a parsimony-based algorithm can reconstruct ancestral sequences. First, to confirm the accuracy and reproducibility of this PGi analysis, I ran PGi program several times using the same analytical parameters and datasets. I found that the detected heterochronic shifts were different each run even though I ran PGi with the same dataset and analytical

parameters. In total, only about 50% of detected heterochronic shifts were replicable. Hence, because of the low accuracy and reproducibility, in this study, I decided to use PGi just as a supportive analysis.

PGi was run four times independently, and by combining these results to a single consensus tree, 207 heterochronic shifts were detected. Similar to the Parsimov results, multiple heterochronic shifts were observed almost all branches (Figure 9). Hence, both Parsimov and PGi analyses implied that multiple heterochronic shifts occurred at almost all branches during fish evolution.

Both of the two methods supported the abundant occurrences of heterochronic shifts over fish phylogenetic tree, but the individual detected shifts varied between two methods. As I described, the results by PGi method were not well replicable. However, the heterochronic shift had actually occurred during fish evolution might be supported by both Parsimov and PGi analyses. I found 53 consensus shifts between two results (Figure 10). Because deltran optimization in Parsimov analysis estimated more shifts in external branches, most of these shifts were located in external branches. Based on the consensus shifts, Gobiiformes, Perchiformes and Salmoniformes were characterized by the earlier shift of hatch (h), eye pigmentation (ep) and blood circulation (bc), respectively. Moreover, most of external blanches belonging to Cypriniformes, Siluriformes, Salmoniformes, Gobiiformes, Cichliformes and Perchiformes retained many consensus shifts, even though they are among most closely related species.

4.7. Simulation-based analyses for distribution patterns of heterochronic shifts

Collaborating with Dr. Tomotaka Matsumoto, we analyzed the distribution patterns of heterochronic shifts. Because a substantial number of heterochronic shifts were detected widely across the fish phylogeny, we wondered whether these shifts might happen rather frequently and be randomly accumulated over the evolutionary history. To address this question, we determined branch length (Figure 11) and took a simulation-based approach. In this section, we only used heterochronic shifts detected by Parsimov method and analyzed the distribution patterns. Given that a heterochronic shift occurs at a random stochastic manner and is neutrally accumulated, we simulated the expected distribution of the number of heterochronic shifts, of which the number was nearly proportional to the phylogenetic branch length (white circles in Figure 12a and 12b). By contrast, the actual distribution of heterochronic shifts detected by the Parsimov analysis was much more constant regardless of the branch length in both acctran and deltran optimizations (black circles in Figure 12a and 12b). Coefficient of variation of the number of heterochronic shifts across the branches also showed smaller value for the experimental dataset than for the simulation data (Figure 12c), indicating that branch-by-branch fluctuations of the number of heterochronic shifts are actually more limited compared with the values expected under simulation. In addition, the number of the phylogenetic branches that harbored no heterochronic shifts was significantly smaller for the experimental dataset than that for the simulation data (Figure 12d). Because inclusion of an extremely long branch could skew the statistical results, we performed the same statistical comparison using only relatively short branches ($\leq$ 50Mya and $\leq$ 20Mya). These analyses again showed similar results

indicating that the number of heterochronic shifts per branch is more constant than the expectation under the assumption of random accumulation (Figure 12c, 12d, Figure 13). Replacing the phylogenic time scale with the generation number basically did not qualitatively affect the results of the analyses (Figure 14 and 15).

The heterochronic shifts of developmental events are sometimes associated to differentiation of terminal phenotypes (Gunter et al., 2014). 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 16). 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.

4.8. Relationship between developmental sequence and duration of embryonic period

In the analysis of sequence orders of event pairs in developmental sequences (Figure 6 and 7), I revealed that hatch (h) was frequently shifted earlier or later than the three events, swim bladder (sb), mouth opening (mo) and caudal fin ray (cfr). Because these three events were expected to directly relate to life strategy, such as swimming and feeding, I hypothesized that earlier shifts of the relative hatch timing in developmental

sequence is related to the short duration of embryonic period. To explore the evolutionary relationship between earlier shifts of hatch and short embryonic period, first, I listed the number of events occurred later than hatch event and duration of embryonic period (hours post fertilization to hatch) (Table 6). Then, I parsimoniously reconstructed the evolutionary histories of earlier shift of hatch (more than 3 event occurred after hatch event) and the short embryonic period (less than 100 hours) (Figure 17) by Mesquite software (Maddison and Maddison 2015). The result estimated that 5 times independent evolution of earlier hatch and 2 to 4 times independent evolution of short embryonic period (Figure 17). Interestingly, Cypriniformes (*C.carpio, B. gonionotus*), Siluriformes (*H. fossilis, H. bidorsalis*), Anabantiformes (*C. striatus, A. testudineus*), and Cichliformes (*A. xiloaensis, C. dimerus*) species belonging to both earlier hatch and short embryonic period lineage, implying that the earlier shift of relative hatch timing and the short duration of embryonic period would be co-evolved in fish phylogeny. Embryogenesis of *C. commersori* proceeds in extremely low temperature (Long and Ballard, 1976) and *L. trewavasa* embryo is protected in their parental month until hatch (Balon, 1977), and these two species showed the earlier shift of hatch and the long embryonic period.

5. DISCUSSION

5.1. Evolutionary rearrangement of fish body plan and evolutionary modularity

The present study provides the empirical evidence that developmental sequences are 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 a blueprint for the common body plan among related species. One reason is probably my wide selection of developmental events; I 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, Workma et al., 2013). Therefore, even though I 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 2008, Kawanishi et al., 2013, Schmidt and 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.

5.2. Heterochronic shifts and fish evolution

The heterochronic shifts detected in this study are widespread all across the fish phylogeny, and the shifts were estimated to occur multiple times in a single branch. In addition, our simulation-based analyses uncovered a certain regularity in the distribution. Namely, the shifts are not randomly accumulated over the evolutionary time, but there

appears to be some force to make the number of shifts constant in individual phylogenetic branches. Teleost fishes would have repeatedly rearranged their developmental sequence by almost every branching event. Thus, it might be possible that the heterochronic shift is a branching-related process. In general, the heterochrony is regarded as one great source of morphological diversity (Gould 1982, Raff and Wray 1989, Hall 1998). In fish lineage, the heterochronic shifts could lead to differentiation of lineage specific phenotypes and would be main driving forces for morphological diversity.

Another interpretation of these results is that 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 6). This limitation probably stems from both developmental and evolutionary constraints in order to the 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.

5.3. High rank changeability and its relation to the evolution

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. The medial finfold is a morphogenetic field for fins. A recent study

showed that the number and morphologies of each medial fin-derived structures, dorsal/anal/caudal fins, had been diverged during fish evolution, and the single morphogenetic field seems to contain multiple evolutionary modules for three distinct fin primodia (Larouche et al., 2017). Thus, it is possible that the three primodia behaved as independent modules during evolution and thereby expanded the temporal range of this event.

5.4. Relative hatch timing and duration of embryonic period

Here, I revealed that the timing of hatch (h) 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 fish survives during the larval stage. In most cases, species with the earlier shift in the relative hatch timing is exposed to the external environment while still immature. Comparing the duration of embryonic period, I hypothesized the co-evolution of the earlier shift of hatch timing and shortening the duration of embryonic period. Importantly, because parsimony analysis estimated this co-evolution independently occurred at least 2 times, thus in some situations, the co-evolution is similarly selected as an advantageous evolutionary change in distinct lineages (Miller and Kendall 2009). However, in unique cases of parental care or environmental condition, such as mouth-brooding and extreme low temperature, the duration of embryonic period seemed to more drastically change than the relative hatch timing, like *C. commersori* and *L. trewavasa*. Similarly, the co-evolutionary phenomena is found in the heterochronic

shifts of the relative birth timing and long pregnancy in the mammalian clade, which have been often related to diversification of mammalian species including human (Keyte and Smith, 2012, Werneburg et al., 2016).

5.5. Variation of developmental sequence and environmental context

There is a common observation that the external temperature affects developmental time frames (Mabee et al., 2000, Schmidt and Starck 2010). Because most fish reproduce by external fertilization and the embryos develop under fluctuating temperatures, temporal shifts of individual developmental events might occur in fish under the natural environment. Indeed, a study reported that the developmental sequence is polymorphic even in one fish species (de Jong et al., 2009). It is possible that fish developmental system is relatively tolerant to a sporadic shift of developmental events in the ontogenetic process. Frequent encounters with such situations may increase the chance that fish has a different developmental sequence, and thereby adopts a new environment in a persistent manner.

5.6. Rank changeability and phylotypic period

When the developmental events were aligned along the ontogenetic sequence, the rank changeability was significantly lower in the middle phase of the early development involving three brains regionalization (tbr), otic placode/primodium (ot), and lens formation (le). These events are typical characteristics of the conserved phylotypic stage determined by the hourglass model (Duboule 1994, Richardson 1995, Irie 2017). The

hourglass model has been gaining increasing support from the recent transcriptome analyses but still lacks sufficient evidence from comparative morphological analyses. Although the relationship between the conservation of the developmental sequences and morphological conformity is not that straightforward, my results support the hourglass model from the morphological point of view. Analyses based on rank changeability as an index of sequence fluctiuation would help us to further discuss why animal body plans are constructed through the phylotypic period.

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FIGURES AND TABLES

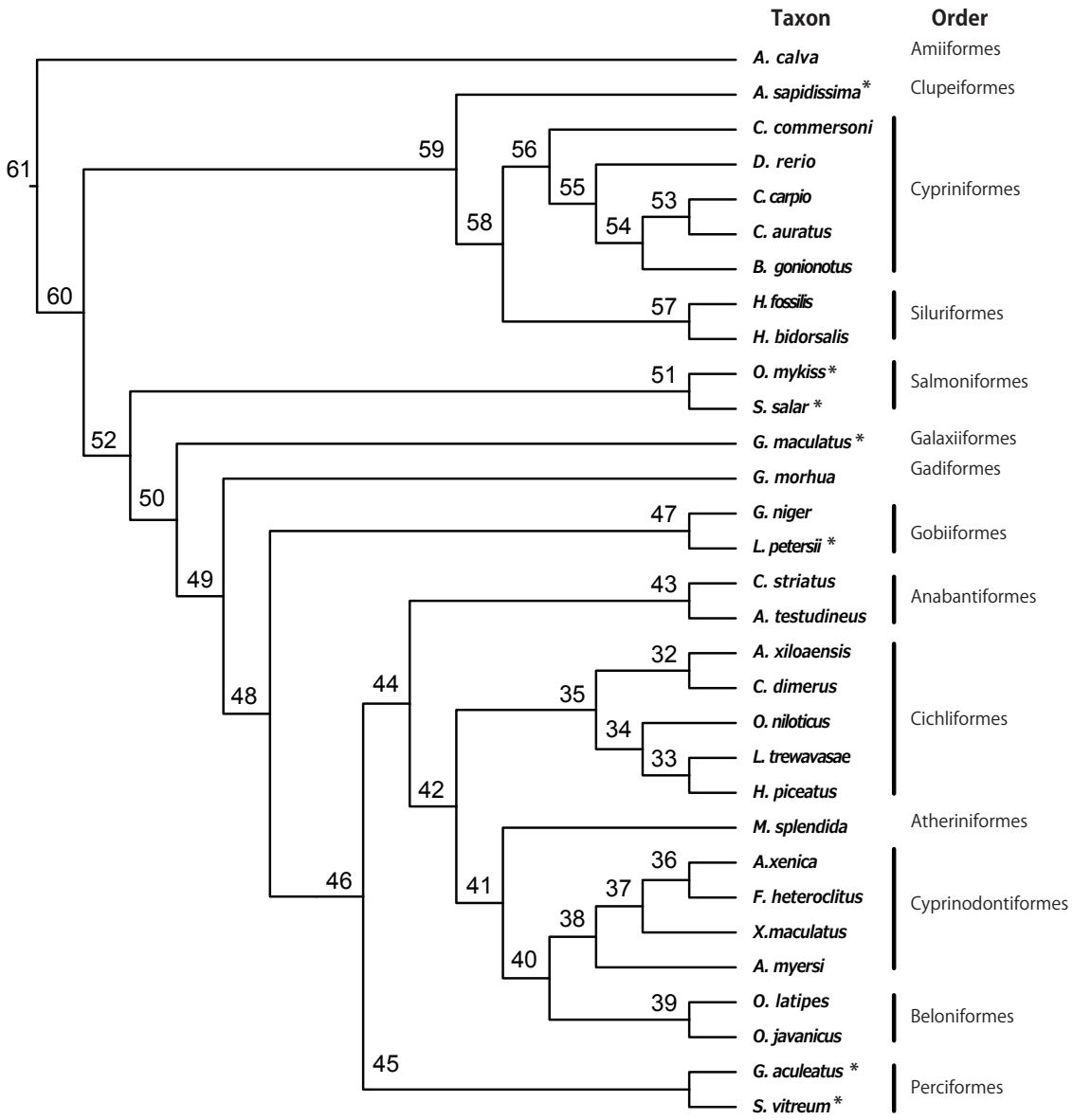

Figure 1. Phylogenetic relationships of the 31 fishes

The phylogenetic tree of the 31 fishes examined in this study. * marks the anadromous fish, while all the others are fresh water fish. Each node was labeled in each number.

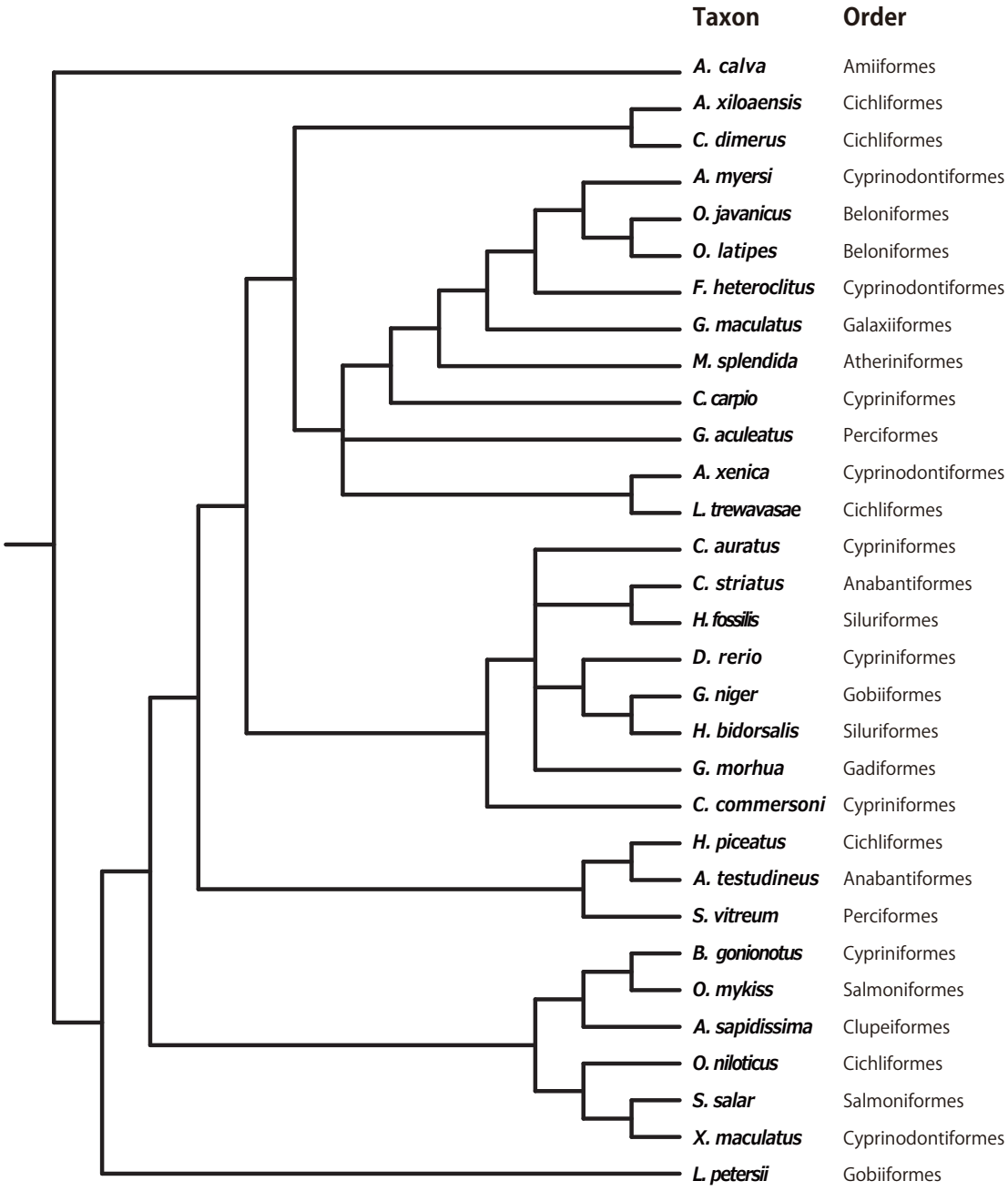

Figure 2. Phylogenetic tree reconstructed by event-pairing matrix

Consensus tree reconstructed by the parsimony analysis of the 190 event-pairing matrix in 31 fish species. Note that the topology of this tree was different from molecular phylogeny presented in Figure 1.

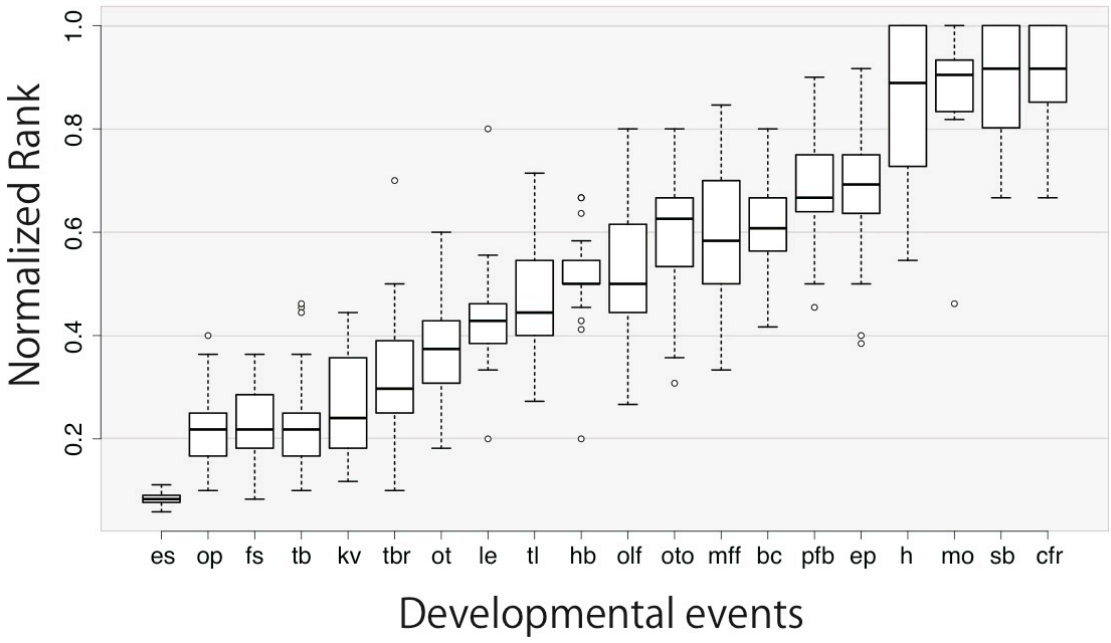

Figure 3. Distribution of ranks of events in the developmental sequence in extant fish

The boxplot shows the statistical distribution of normalized ranks for individual developmental events obtained from the extant in-group 30 fish data. The developmental events are horizontally aligned from left to right according to average ranks in the extant fish sequences.

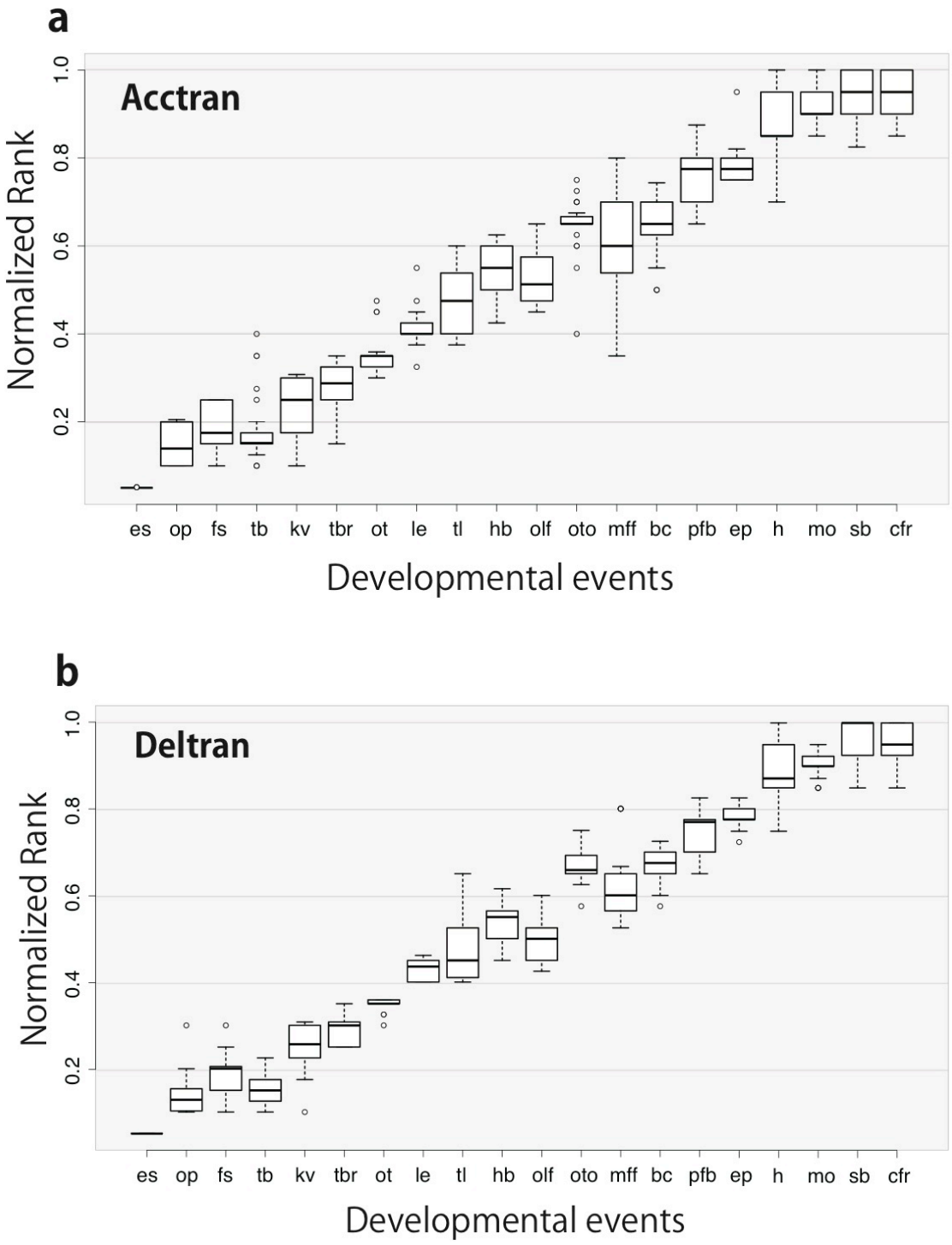

Figure 4. Distribution of ranks of events in the developmental sequence

The boxplot shows the statistical distribution of normalized ranks for individual developmental events obtained from reconstructed ancestral developmental sequences

by acctran (a) and deltran (b) optimizations. The developmental events are horizontally aligned from left to right according to average ranks in the extant fish sequences. In the ancestral sequences (a, b), the average sequence is reversed between first somite (fs) and tail bud (tb), between heart beats (hb) and olfactory vesicle/pit/placode (olf), and between blood circulation (bc) and otolithes (oto). An additional inversion is observed between swim bladder (sb).

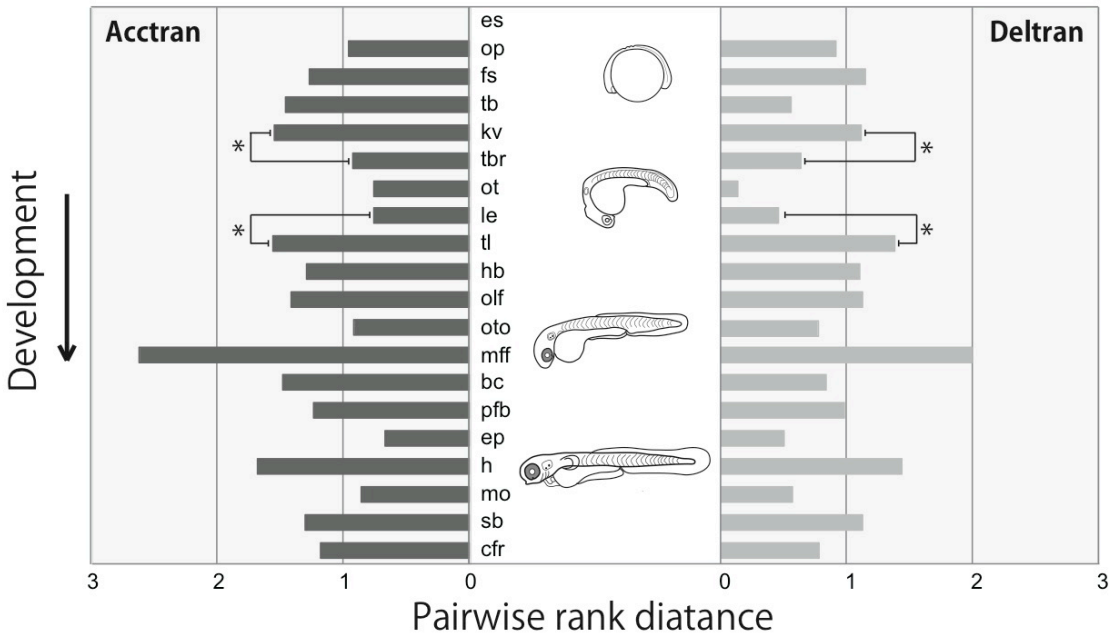

Figure 5. Rank changeability of individual developmental events

The variation of the ranks is shown as the average value of pairwise rank distances, which are calculated from all the pairs of ancestral developmental sequences reconstructed under acctran (left) and deltran (right) optimizations. The events are arranged along the standard ontogenic time frame defined by the average developmental sequence in extant fish (Figure1) from top to bottom. *significant differences ($P<0.05$) by Mann-Whitney U-test when comparing the values of Kupffer's vesicle (kv) and three brain regionalization (tbr) and those of lens formation (le) and tail lift from yolk (tl).

	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 6. Frequency of sequence reversal between the developmental events

The event sequence matrix represents all the pairwise combinations of developmental events. The number shows the percentage of the sequences in which the row event occurs later than the column event, and was calculated from the dataset of extant 30 fishes excluding the missing event data. The individual cells are differently heatmap color-coded depending on the percentage.

a

Acctran

	es	op	fs	tb	kv	tbr	ot	le	tl	hb	olf	oto	mff	bc	pfb	ep	h	mo	sb
op	0%																		
fs	0%	30%																	
tb	0%	27%	53%																
kv	0%	13%	30%	27%															
tbr	0%	0%	3%	10%	37%														
ot	0%	0%	0%	10%	0%	3%													
le	0%	0%	0%	3%	0%	0%	7%												
tl	0%	0%	0%	0%	0%	0%	7%	23%											
hb	0%	0%	0%	0%	0%	0%	0%	0%	27%										
olf	0%	0%	0%	0%	0%	0%	0%	0%	20%	57%									
oto	0%	0%	0%	0%	0%	0%	3%	3%	7%	3%	7%								
mff	0%	0%	0%	0%	0%	0%	3%	3%	3%	37%	13%	57%							
bc	0%	0%	0%	0%	0%	0%	0%	0%	10%	0%	17%	33%	30%						
pfb	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	10%	20%	3%					
ep	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	10%	0%	33%				
h	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	3%	0%	10%	7%			
mo	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	3%	37%		
sb	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	3%	27%	30%	
cfr	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	33%	27%	47%

b

Deltran

	es	op	fs	tb	kv	tbr	ot	le	tl	hb	olf	oto	mff	bc	pfb	ep	h	mo	sb
op	0%																		
fs	0%	20%																	
tb	0%	17%	57%																
kv	0%	7%	20%	3%															
tbr	0%	3%	3%	0%	33%														
ot	0%	0%	0%	0%	0%	3%													
le	0%	0%	0%	0%	0%	0%	0%												
tl	0%	0%	0%	0%	0%	0%	0%	37%											
hb	0%	0%	0%	0%	0%	0%	0%	0%	17%										
olf	0%	0%	0%	0%	0%	0%	0%	0%	27%	60%									
oto	0%	0%	0%	0%	0%	0%	0%	0%	3%	0%	0%								
mff	0%	0%	0%	0%	0%	0%	0%	0%	0%	20%	3%	57%							
bc	0%	0%	0%	0%	0%	0%	0%	0%	3%	0%	0%	27%	20%						
pfb	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	17%	20%	3%					
ep	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	20%	0%	3%				
h	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	3%	3%			
mo	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	40%		
sb	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	23%	20%	
cfr	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	30%	10%	43%

Figure 7. Sequence orders of event pairs in ancestral developmental sequences

The percentage of the sequences in which the row event occurs later than the column event calculated from the ancestral developmental sequences reconstructed under acctran (a) and deltran (b) optimizations.

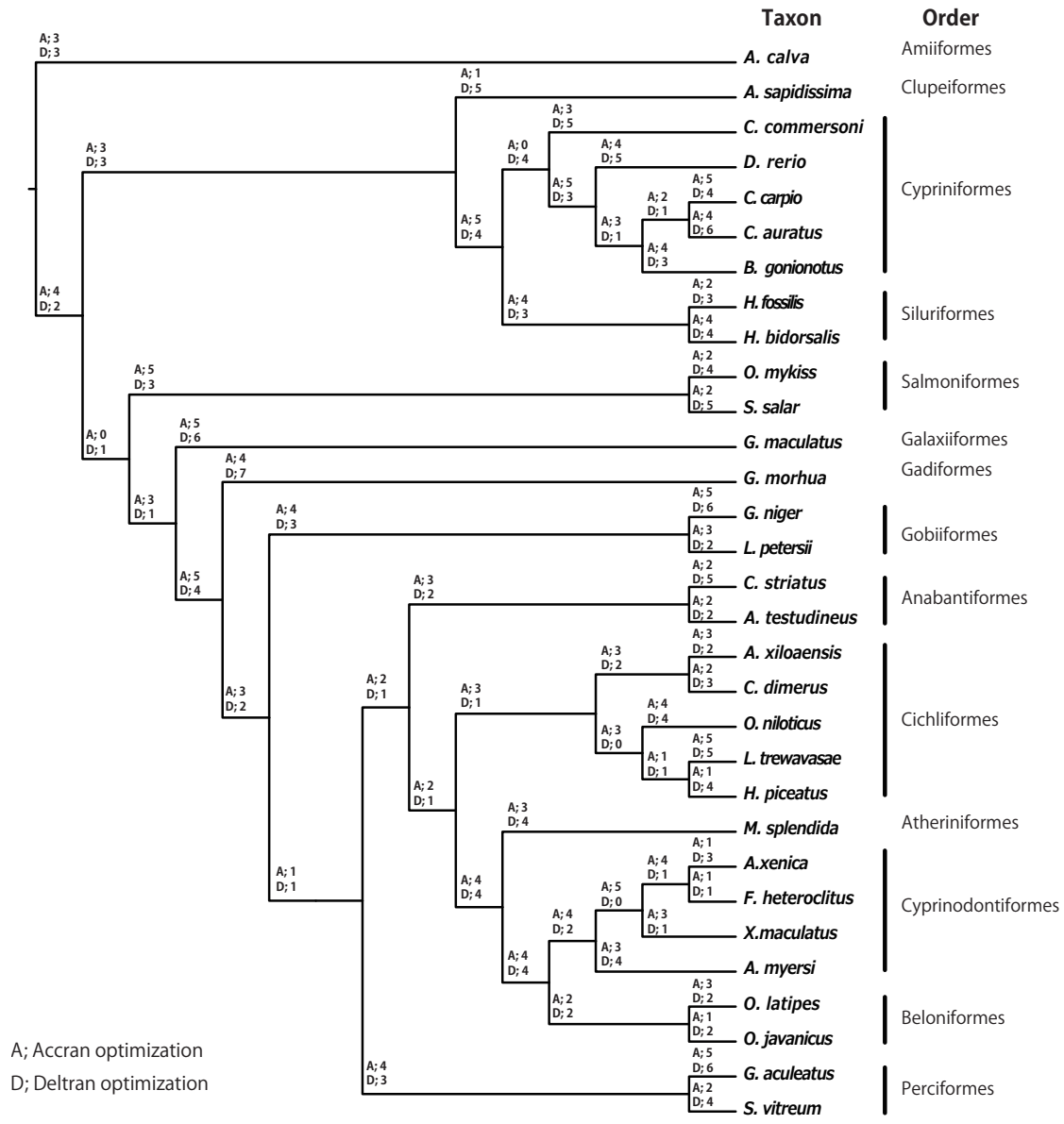

Figure 8. The number of detected heterochronic shifts by Parsimov analysis

The number of detected heterochronic shift in each branch was mapped on fish molecular phylogeny. A and D indicated the acctran and deltran optimization, respectively. Note that there were no branches with no heterochronic shifts.

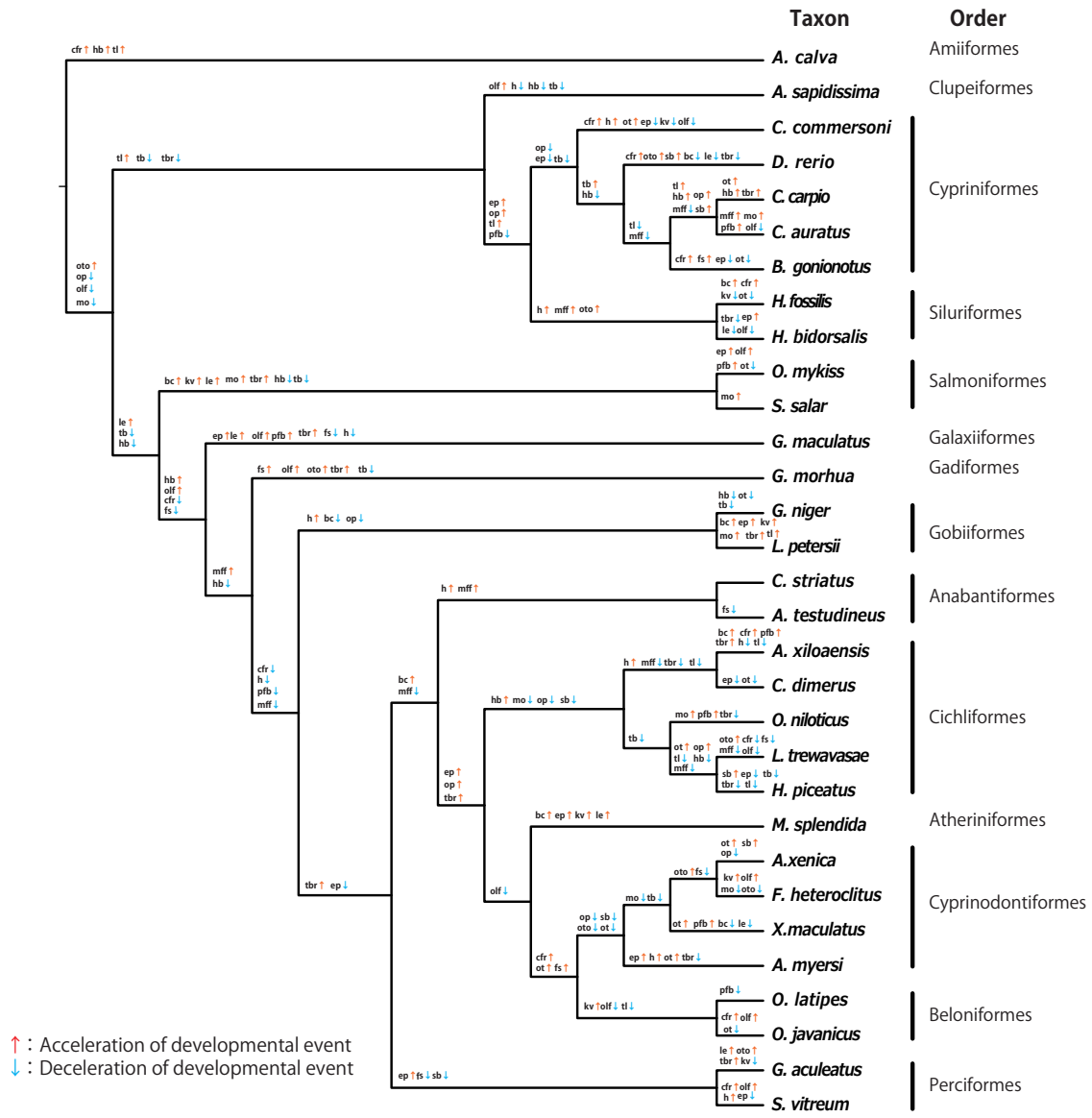

Figure 9. Distribution of heterochronic shifts in the fish phylogeny detected by PGi anlysis

The heterochronic shifts detected by PGi analysis were mapped onto fish phylogeny. Abbreviations of developmental events are following Table 1. Note that there was only one branch with no heterochronic shift. The red arrow indicates acceleration of event shift and blue arrow indicates deceleration of event shift.

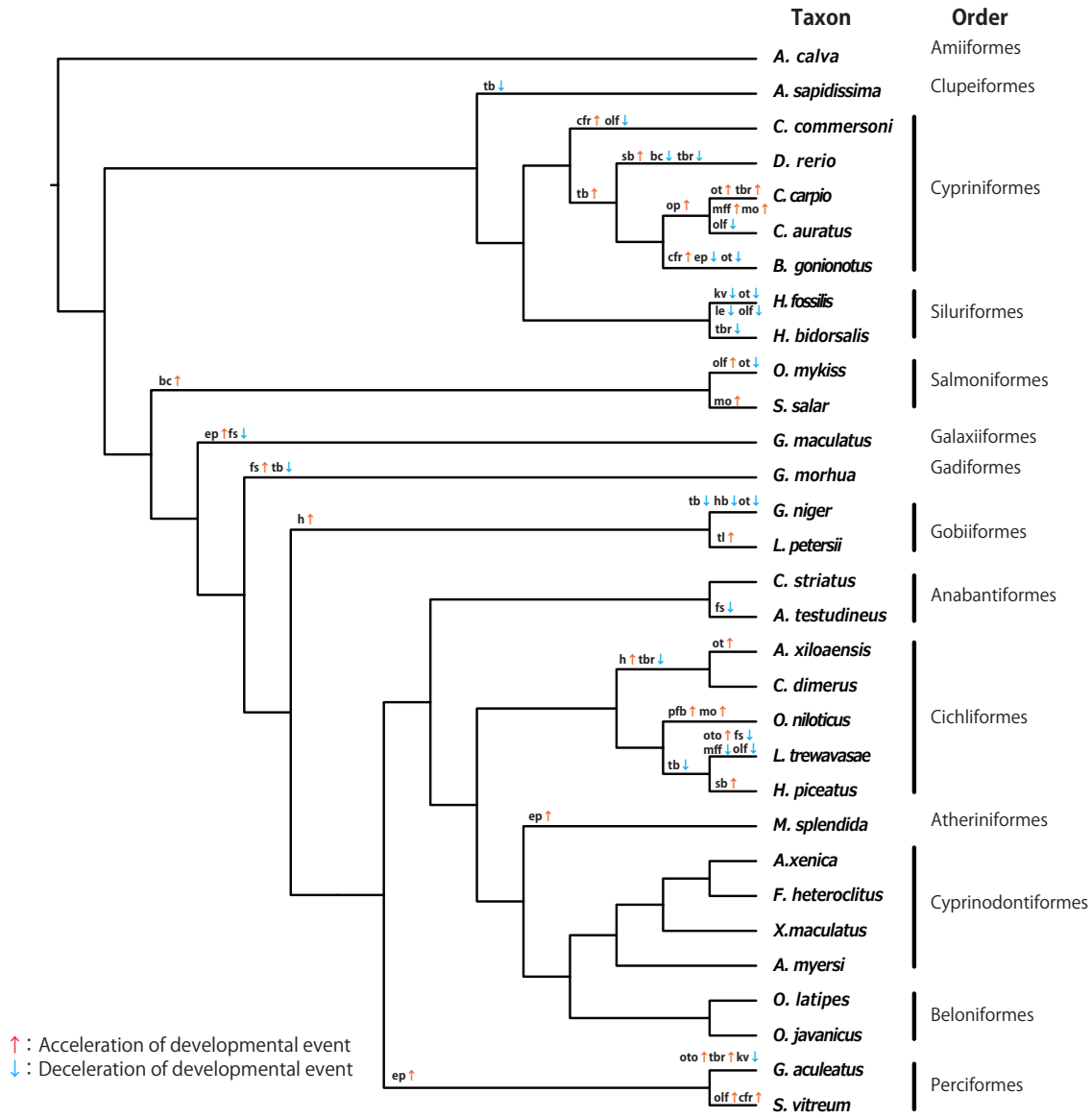

Figure 10. Distribution of consensus heterochronic shifts detected by both Parsimov and PGi analyses

The consensus heterochronic shifts detected by both Parsimov and PGi analyses were mapped onto fish phylogeny. Abbreviations of developmental events are following Table 2. The red arrow indicates acceleration of event shift and blue arrow indicates deceleration of event shift.

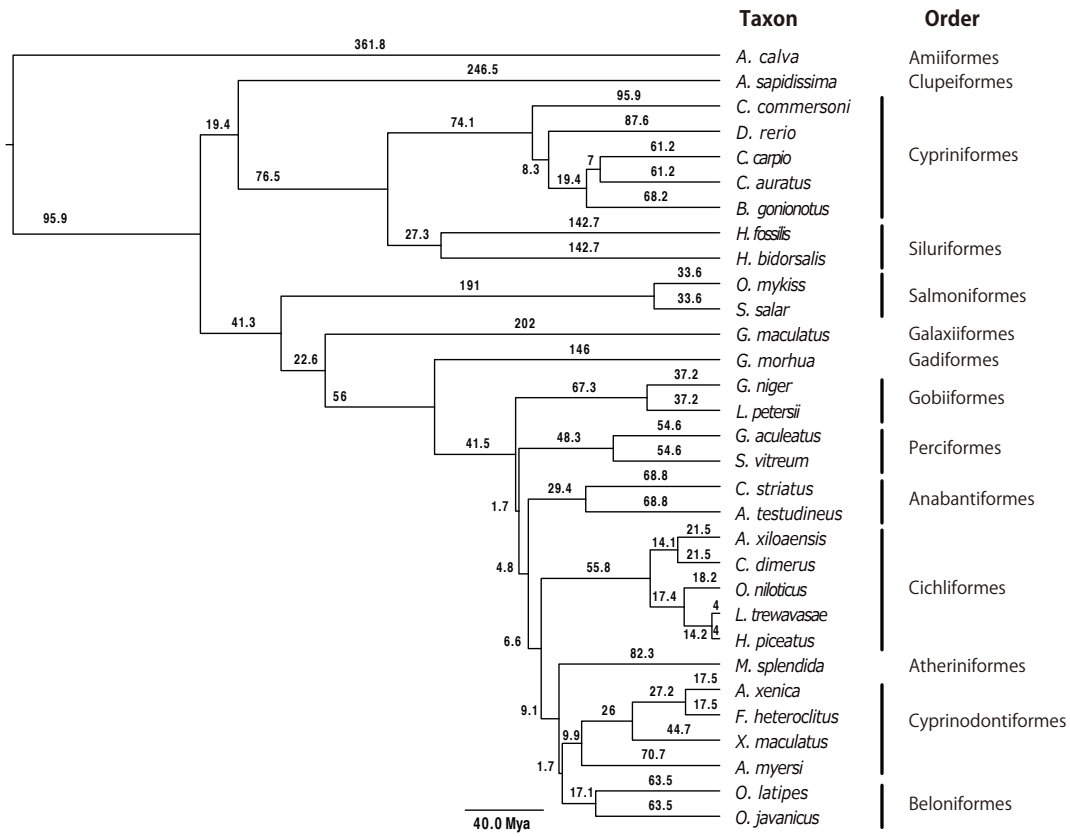

Figure 11. Branch length of the 31 fishes phylogenetic tree

The numbers aside the branches indicate the divergent times (Mya).

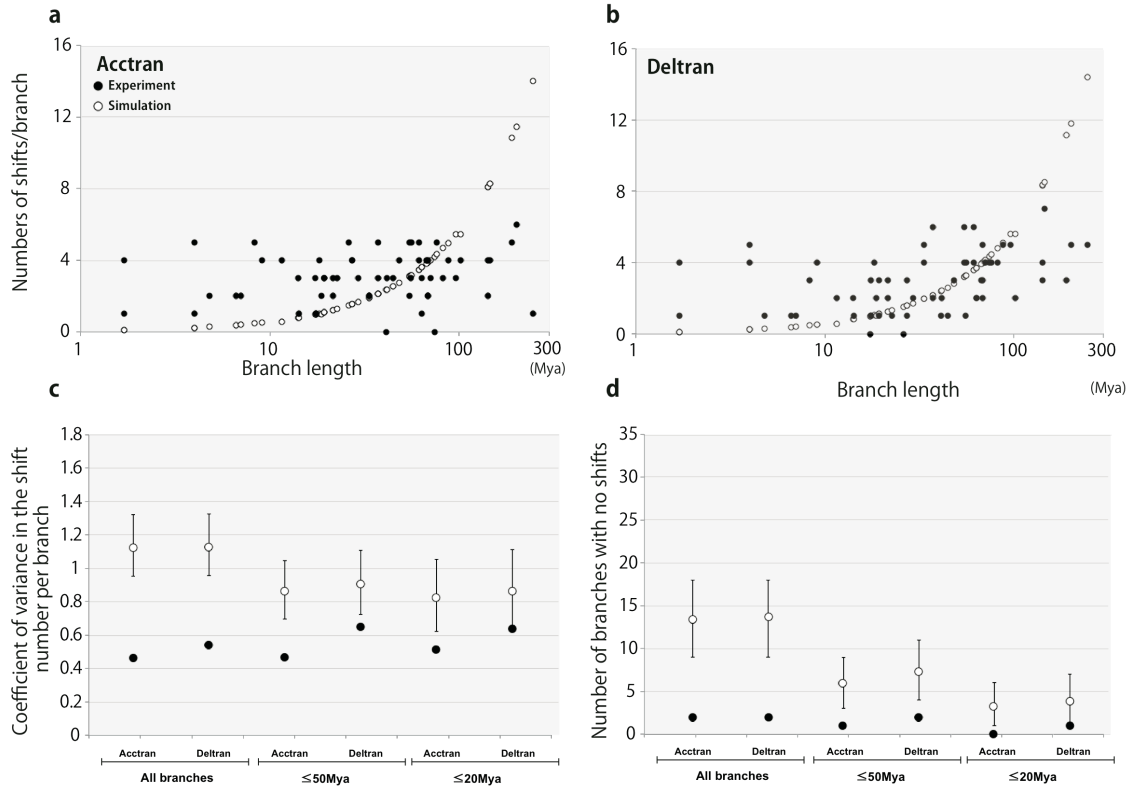

Figure 12. Distribution of heterochronic shifts in the fish phylogeny

(a,b) The relationship between the phylogenetic branch length and the number of herterochronic shifts detected from the extant and ancestral developmental sequences (black circle) and theoretically estimated by simulation (open circle) under acctran (a) and deltran (b) optimizations. In the simulation, the branch length and the numbers of shifts are highly correlated (Spearman's rank correlation coefficients; 0.9995 (acctran), 0.9995 (deltran)) (c) The coefficient of variance for the number of heterochronic shifts in each branch. The black and open circles show the experimental and simulated values, respectively. The vertical bars indicate 95% confident intervals for the simulated value. The analysis was conducted with three different branch categories: all branches, and the branches shorter than 50 and 20 million years (Mys). (d) The number of branches with no heterochronic shifts calculated from experimental (black circle) and simulation data

(open circle) in three different branch length categories. Vertical bars indicate 95% confident intervals of the simulated value.

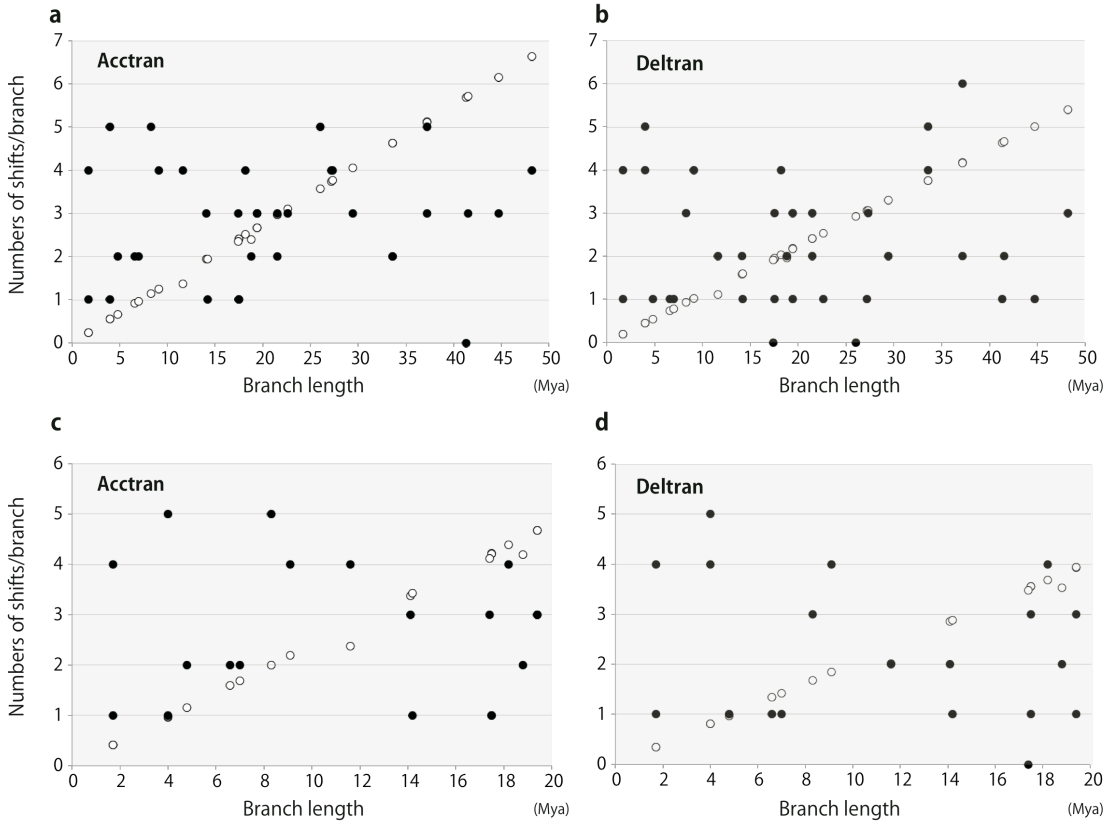

Figure 13. Distribution of herterochronic shifts in shorter branch categories

The relationship between the phylogenetic branch length and the number of heterochronic shifts in the extant and ancestral developmental sequences (black circle) and theoretically estimated by the simulation (open circle) under acctran (a, c) and deltran (b, d) optimizations. Only the branches shorter than 50 Mya (a, b) and 20 Mya (c, d) are represented.

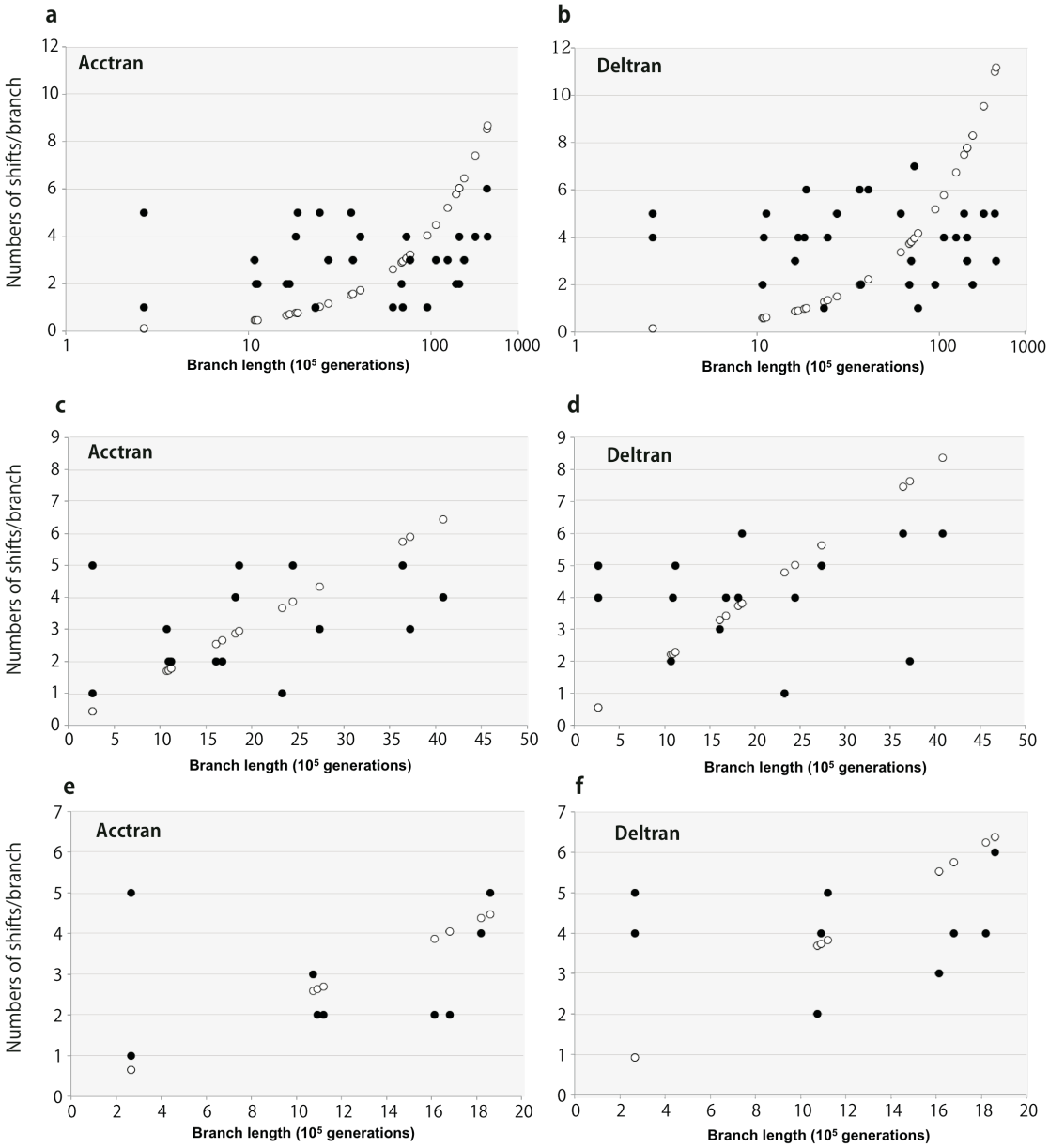

Figure 14. Distribution of heterochronic shifts on the branches scaled by the generation number

The relationships between the generation number and the number of heterochronic shifts for the experimental data (black circle) and simulation data (open circle) under acctran (a, c, e) and deltran (b, d, f) optimizations. The horizontal axis is scaled by the generation number considering the average generation time of each species. The branch

are categorized into all branch (a, b), the branches shorter than 50*10 generations (c, d) and 20*10 generations (e, f).

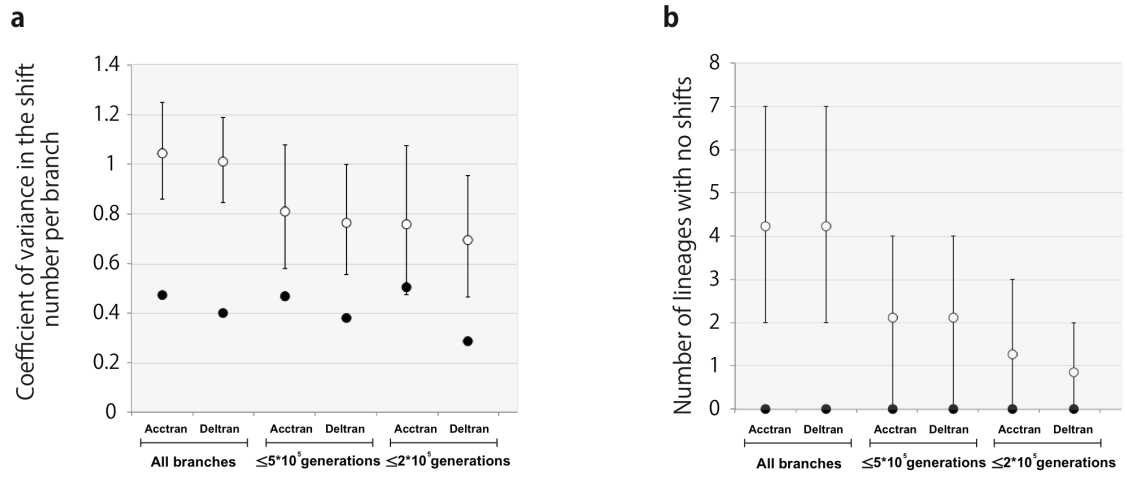

Figure 15. Statistical comparisons of heterochronic shifts on the branches scaled by the generation number

(a) The coefficient of variance for the number of heterochronic shifts in each branch (Figure 5c) rescaled by the generation number as phylogenetic time. (b) The number of branches with no heterochronic shifts (Figure 5d) rescaled by the generation number as phylogenetic time. The labels and marks are the same as those in Figure 5c and d.

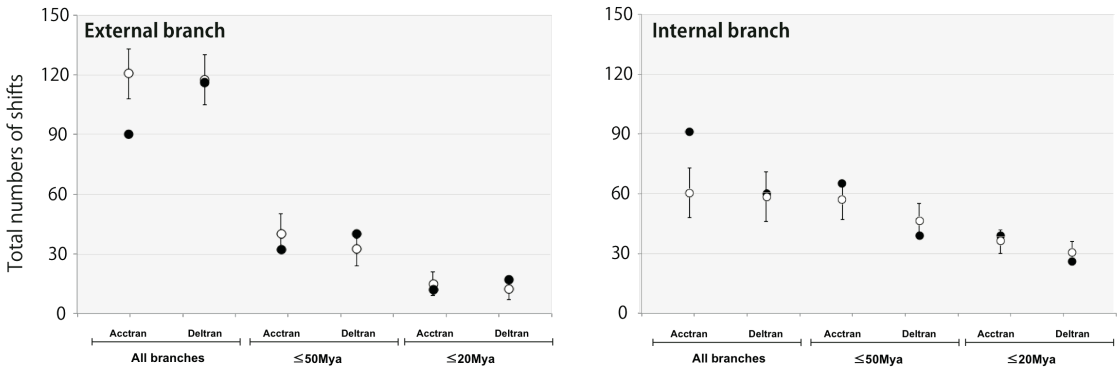

Figure 16. Comparisons of the number of heterochronic shifts in external and internal branches

The number of heterochronic shifts detected in the external (a) and internal (b) branches calculated from experimental (black circle) and simulation data (open circle). The branches are categorized into tree groups according to their lengths. Vertical bars indicate 95% confident intervals of the simulated value.

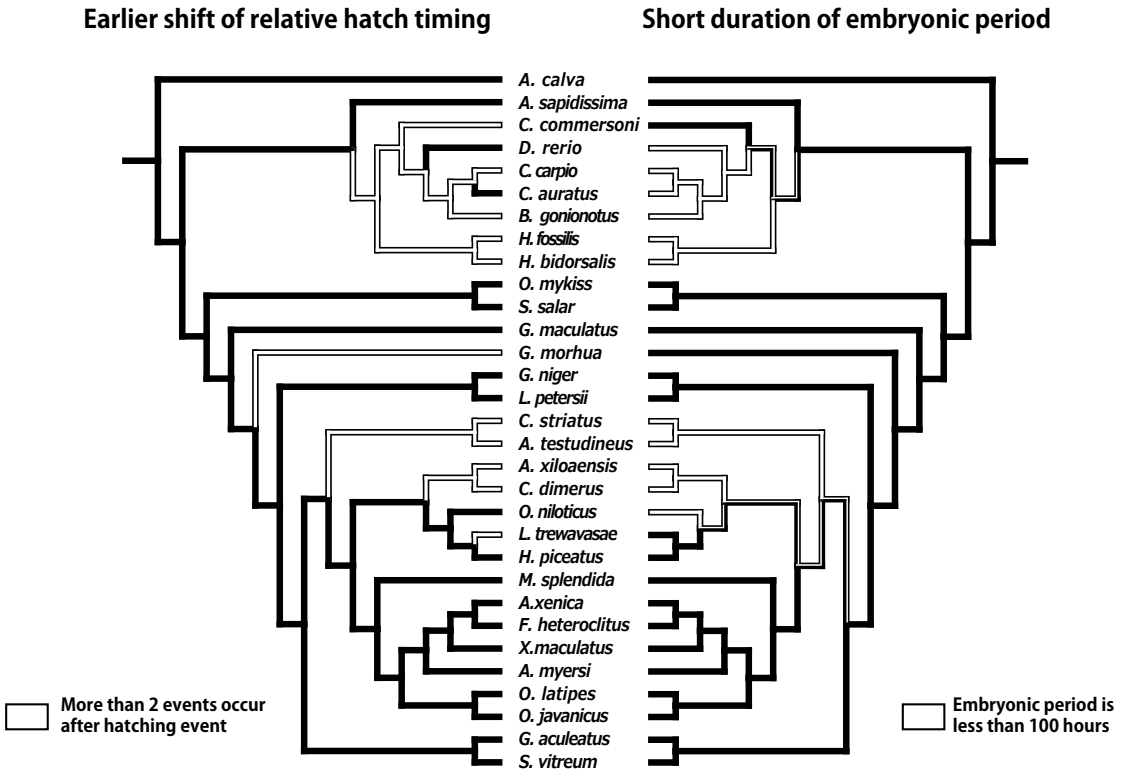

Figure 17. Evolutionary history of event shifts and duration of embryonic period

Evolutionary histories of earlier shift in relative hatch timing (left tree) and shortening duration of embryonic period (right). These ancestral states were parsimoniously reconstructed by Mesquite software. In these phylogeny, more than 2 events occurred after hatch events was marked white line in the left, and less than 100 hours embryonic period was marked white line in the right.

Branch name	Branch length (Mya)	Reference
Labeotropheus trewavasae	**4.0**	Sanciangco et al., 2015
Haplochromis piceatus	**4.0**	Sanciangco et al., 2015
Adinia xenica	**17.5**	Near et al., 2013
Fundulus heteroclitus	**17.5**	Near et al., 2013
Oreochromis niloticus	**18.2**	Sanciangco et al., 2015
Amphilophus xiloaensis	**21.5**	Friedman et al., 2013
Cichlasoma dimerus	**21.5**	Friedman et al., 2013
Oncorhynchus mykiss	**33.6**	Ma et al., 2013
Salmo salar	**33.6**	Ma et al., 2013
Gobius niger	**37.2**	Near et al., 2012
Leucopsarion petersii	**37.2**	Near et al., 2012
Xiphophorus maculatus	**44.7**	Near et al., 2013
Gasterosteus aculeatus	**54.6**	Near et al., 2013
Stizostedion vitreum	**54.6**	Near et al., 2013
Cyprinus carpio	**61.2**	Li et al., 2013
Carassius auratus	**61.2**	Li et al., 2013
Oryzias latipes	**63.5**	Setiamarga et al., 2009
Oryzias javanicus	**63.5**	Setiamarga et al., 2009
Barbodes gonionotus	**68.2**	Li et al., 2013
Channa striatus	**68.6**	Near et al., 2013
Anabas testudineus	**68.6**	Near et al., 2013
Austrofundulus myersi	**70.7**	Near et al., 2013
Melanotaenia splendida	**82.3**	Near et al., 2013
Danio rerio	**87.6**	Zhao et al., 2015
Catostomus commersoni	**95.9**	Near et al., 2012
Heterobranchus bidorsalis	**142.7**	Nakatani et al., 2011
Heteropneustes fossilis	**142.7**	Nakatani et al., 2011
Gadus morhua	**146.0**	Near et al., 2012
Galaxias maculatus	**202.0**	Near et al., 2012
Alosa sapidissima	**246.5**	Near et al., 2012
Amia calva	**361.8**	Near et al., 2012

Table 1. List of branch length and references

Taxon	Common name	Reference	Ranks of events																		
			es	op	fs	tb	kv	tbr	ot	le	tl	hb	olf	oto	mff	bc	pfb	ep	h	mo	sb	cfr
Amia calva	Bowfins	Ballard, 1986	1	2	2	2	?	3	4	5	5	5	5	8	6	7	7	7	10	8	9	9
Alosa sapidissima	American shad	Shardo, 1995	1	4	2	5	?	3	5	5	6	7	5	7	6	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	7	6	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	8	11	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 and 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	9	7	7	7	8	12	10	?	11
Salmo salar	Atlantic salmon	Pelluet, 1944, Gorodilov, 1996	1	3	2	6	2	?	4	5	?	7	8	9	8	8	9	10	12	6	13	11
Galaxias maculatus	Common galaxias	Benzie, 1968	?	1	2	1	2	1	3	2	5	2	3	8	7	?	6	4	10	10	?	9
Gadus morhua	Atlantic cod	Hall et al., 2004	1	2	1	3	3	3	4	6	?	7	5	5	4	8	8	9	10	12	11	11
Gobius niger	Black goby	Ballard, 1969	1	2	2	3	3	6	5	4	4	8	?	7	5	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 and Balon, 1979	1	2	3	2	3	4	4	5	6	7	4	8	6	9	11	10	13	14	15	12
Channa striatus	Striped snakehead	Marimuthu and Haniffa, 2007	1	2	2	2	4	3	3	4	4	5	5	6	4	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 and Guerrero, 2000	1	4	3	2	?	5	5	5	6	5	?	6	8	6	9	8	7	10	?	10
Oreochromis niloticus	Nile tilapia	Fujimura and Okada, 2007	1	3	2	3	?	4	5	6	5	6	6	8	7	8	8	9	10	10	12	11
Labeotropheus trewavasae	Scrapermouth mbuna	Balon, 1977	1	2	4	3	?	2	3	5	5	6	11	5	9	7	9	8	10	12	14	13
Haplochromis piceatus	Victoria cichlids	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	?	8	11	7	9	5	12	11	10	13
Adinia xenica	Diamond killfish	Cunningham and Balon, 1985	1	2	3	?	3	2	3	5	?	6	8	4	9	7	8	9	13	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	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	?	9	11	8	9	10	14	13	11	12
Oryzias latipes	Japanese ricefish	Iwamatsu, 2004	1	3	4	?	2	5	4	6	9	7	13	8	12	8	10	11	17	15	14	16
Oryzias javanicus	Javanese ricefish	Iwamatsu and Hirata, 1984	1	3	4	?	2	5	5	6	9	7	7	8	?	8	9	10	14	13	11	12

Table 2. Temporal orders of developmental events in the 31 fishes

The temporal sequence of developmental events was extracted from the reference 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 brains regionalization, and tl: tail lift from yolk. The ranks of missing data are marked by "?".

Acctran	es	op	fs	tb	kv	tbr	ot	le	tl	hb	olf	oto	mff	bc	pfb	ep	h	mo	sb	cfr
node32	1	2	5	3.5	3.5	7	6	8	9	10.5	10.5	12	15	13	17	16	14	18	20	19
node33	1	2	3.5	5.5	3.5	5.5	7	9	8	11	10	13	13	13	15.5	15.5	17	18	20	19
node34	1	2	3.5	5	3.5	6	7	9	8	11	10	13	13	13	15.5	15.5	17	18	20	19
node35	1	2	5	3.5	3.5	6.5	6.5	9	8	11	10	12.5	14	12.5	16	15	17	18	20	19
node36	1	2	5	7	4	3	6	8	11	9	11	13	15.5	11	14	15.5	20	19	18	17
node37	1	2	3	7	4	5	6	8.5	10	8.5	12	14	15.5	11	13	15.5	20	19	18	17
node38	1	3.5	5	3.5	2	6	7	8.5	11.5	8.5	11.5	13	16	10	14	15	20	19	17	18
node39	1	3	5	4	2	6.5	6.5	8	12	9	13	11	15.5	10	14	15.5	20	19	17	18
node40	1	3	5	4	2	6.5	6.5	8	12	9	12	12	16	10	14	15	20	19	17	18
node41	1	2	5	3.5	3.5	6.5	6.5	8	9	10	12	13	16	11	14	15	19	18	17	20
node42	1	2	5	3.5	3.5	6.5	6.5	8.5	8.5	10	11	13	14	12	16	15	17	18.5	18.5	20
node43	1	2	4	3	5	6.5	6.5	9.5	8	11	12	14	9.5	13	16.5	19	15	18	16.5	20
node44	1	2	5	3	4	6.5	6.5	8	9	12	10.5	14	10.5	13	16	15	17	18.5	18.5	20
node45	1	2.5	4.5	2.5	4.5	6.5	6.5	8	10.5	12	9	13	10.5	14	16	15	17	18	19.5	19.5
node46	1	2	4	3	5	6.5	6.5	8	10	12	9	13	11	14	15.5	15.5	17	18.5	18.5	20
node47	1	2.5	4	2.5	5	6.5	6.5	8	9	12	10.5	13	10.5	14	16	15	18	19	17	20
node48	1	2.5	4	2.5	5	6	7	8	9.5	12	9.5	13	11	14	15.5	15.5	17	18.5	18.5	20
node49	1	2	3.5	3.5	5.5	5.5	7	8	9.5	12	9.5	13	11	14	15	16	17	20	19	18
node50	1	3	3	3	6	5	7	8	11	10	9	13	12	14.5	14.5	16	18	19.5	19.5	17
node51	1	4	2.5	8	2.5	5	6.5	6.5	9.5	11	9.5	15	12	13	14	16	19	17	20	18
node52	1	4	2	3	6	5	7	8	11	10	9	13.5	12	13.5	15	16	19	17	20	18
node53	1	4	3	2	6	5	9	7.5	7.5	11	10	13	13	13	15.5	15.5	17	18	19	20
node54	1	4	3	2	6	5	9	7.5	7.5	11	10	13	13	13	15.5	15.5	17	18	19	20
node55	1	4	3	2	6	5	7	9	8	10	11.5	13	11.5	14	16	15	17	18	19	20
node56	1	4	2	3	6	5	7	9	8	11	12	13	10	14	16	15	17	18	20	19
node57	1	3	3	3	5	6	9.5	11	9.5	12.5	12.5	8	7	14	17.5	15	16	19	20	17.5
node58	1	4	2	3	6	5	7	8.5	8.5	11.5	11.5	13	10	14	16	15	17	18	20	19
node59	1	4	2	3	6	5	7	8	10.5	12	9	13	10.5	14	15	16	18	17	19.5	19.5
node60	1	4	2	3	6	5	7	8	11	10	9	13.5	12	13.5	15	16	19	17	20	18
node61	1	3	3	3	6	5	7	8	9.5	11	9.5	14.5	12	14.5	13	16	20	17	18.5	18.5

Table 3a.

Deltran	es	op	fs	tb	kv	tbr	ot	le	tl	hb	olf	oto	mff	bc	pfb	ep	h	mo	sb	cfr
node32	1	2.5	4	2.5	5	7	6	8.5	8.5	10.5	10.5	13	13	13	16.5	16.5	15	18	20	19
node33	1	2	3	4.5	4.5	6	7	9	8	11	10	13	13	13	15.5	15.5	17	18	20	19
node34	1	2.5	4	2.5	5	6	7	9	8	11	10	13	13	13	15.5	15.5	17	18	20	19
node35	1	2.5	4	2.5	5	6	7	9	8	11	10	13	13	13	15.5	15.5	17	18	20	19
node36	1	2	6	3	4	5	7	8	10.5	9	10.5	13	16	12	14	15	20	19	18	17
node37	1	2	5	3.5	3.5	6	7	8	10.5	9	10.5	13	16	12	14	15	20	19	17	18
node38	1	2	5	3.5	3.5	6	7	8	10.5	9	10.5	13	16	12	14	15	20	19	17	18
node39	1	3.5	5	3.5	2	6.5	6.5	8	13	9	10	11.5	16	11.5	14	15	20	18	17	19
node40	1	2	5	3.5	3.5	6	7	8	10.5	9	10.5	13	16	12	14	15	20	18	17	19
node41	1	2	5	3.5	3.5	6	7	8	9	11	10	12.5	16	12.5	14.5	14.5	19	18	17	20
node42	1	2.5	4	2.5	5	6	7	9	8	11	10	13	13	13	15.5	15.5	17	18	19.5	19.5
node43	1	2.5	4	2.5	5	6.5	6.5	9	8	12	10	13.5	11	13.5	15.5	15.5	17	18.5	18.5	20
node44	1	2.5	4	2.5	5	6	7	9	8	12	10	13.5	11	13.5	15.5	15.5	17	18	19.5	19.5
node45	1	2	4	3	5	6	7	8.5	10	12	8.5	13	11	14	16	15	17	18	19.5	19.5
node46	1	2.5	4	2.5	5	6	7	8.5	10	12	8.5	13.5	11	13.5	15.5	15.5	17	18	19.5	19.5
node47	1	2.5	4	2.5	5	6	7	9	9	12	9	13	11	14	15.5	15.5	17.5	17.5	19	20
node48	1	2.5	4	2.5	5	6	7	8.5	10	12	8.5	13	11	14	15	16	17	18	19.5	19.5
node49	1	2	3.5	3.5	5.5	5.5	7	8.5	10	11.5	8.5	13	11.5	14	15	16	17	18	19.5	19.5
node50	1	3	3	3	6	5	7	8.5	10.5	10.5	8.5	15	12	13.5	13.5	16	18.5	17	20	18.5
node51	1	6	2	3	4	5	7	8.5	10.5	10.5	8.5	15	12	13	14	16	19	17	20	18
node52	1	3	3	3	6	5	7	8.5	10.5	10.5	8.5	15	12	13.5	13.5	16	19	17	20	18
node53	1	4	3	2	6	5	7	9	8	10	11	13	13	13	15.5	15.5	17	18	20	19
node54	1	4	2.5	2.5	6	5	7	9	8	10	11	13	13	13	15.5	15.5	17	18	20	19
node55	1	4	2.5	2.5	6	5	7	9	8	10	11.5	13	11.5	14	15.5	15.5	17	18	20	19
node56	1	4	2	3	6	5	7	8.5	8.5	10.5	12	13	10.5	14	15.5	15.5	17	18	20	19
node57	1	3	3	3	5.5	5.5	7	9	8	11	11	13	11	14	15	16	17	18	19.5	19.5
node58	1	3.5	2	3.5	6	5	7	9	8	11	11	13.5	11	13.5	15	16	17	18	19.5	19.5
node59	1	3	3	3	6	5	7	8.5	8.5	11	10	13.5	12	13.5	15	16	18	17	19.5	19.5
node60	1	3	3	3	6	5	7	8	9.5	11	9.5	15	12	13.5	13.5	16	19	17	20	18
node61	1	3	3	3	6	5	7	8	9.5	11	9.5	14.5	12	14.5	13	16	20	17	18.5	18.5

Table 3b.

Table 3. Ancestral developmental sequences reconstructed by event-pairing method

Event ranks in ancestral node were listed. The ancestral sequence was reconstructed in acctran (a) and deltran (b) optimizations. The node numbers were shown in Figure 1.

Node	Acctran	Deltran	Conserved
34 → 33	**Event** tb moved L relative to ot, op, tbr	**Event** tb moved L relative to ot, op, tbr	**Event** tb moved L relative to ot, op, tbr
35 → 32	Twins (oto, tl), **Event** h moved E relative to ep, mff, pfb, **Event** tbr moved L relative to hb, le	**Event** h moved E relative to ep, pfb , **Event** tbr moved L relative to hb, le, ot	**Event** h moved E relative to ep, pfb, **Event** tbr moved L relative to hb, le, ot
35 → 34	Twins (fs, tb) (tbr, ot), **Event** mff moved E relative to bc, ep, oto		
37 → 36	Twins (le, hb) (olf, tl) (oto, pfb) , **Event** fs moved L relative to op, tbr	Twins (cfr, sb)	
38 → 37	Twins (cfr, sb) (mff, ep), **Event** kv moved L relative to op, tbr, **Event** tb moved L relative to fs, ot, tbr, **Event** tl moved E relative to bc, oto		
40 → 38	Twins (fs, op) (hb, le) (pfb, oto) (tbr, ot)	Twins (bc, oto) (cfr, mo)	
40 → 39	Twins (mff, olf)	Twins (ot, tbr)	Twins (ot, tbr)

	(oto, bc)	, **Event** tl moved L relative to bc, oto	
41 →40	Twins (kv, op) (pfb, ep), **Event** cfr moved E relative to h, mo , **Event** tl moved L relative to bc, hb, oto	Twins (cfr, h) (le, olf) (pfb, ep) , **Event** tl moved L relative to bc, hb	Twins (pfb, ep), **Event** tl moved L relative to bc, hb
42 → 35	Twins (fs, op) , **Event** olf moved E relative to bc, le , **Event** sb moved L relative to cfr, mo	Twins (cfr, sb)	
42 → 41	Twins (bc, oto) (le, tl), **Event** mff moved L relative to ep, pfb, **Event** olf moved L relative to hb, pfb	Twins (le, tl) , **Event** kv moved E relative to fs, tb , **Event** mff moved L relative to bc, ep, oto, pfb , **Event** sb moved E relative to cfr, mo	Twins (le, tl), **Event** mff moved L relative to ep, pfb,
44 → 42	Twins (kv, tb) , **Event** mff moved L relative to bc, hb, oto, tl	**Event** mff moved L relative to bc, hb, oto	**Event** mff moved L relative to bc, hb, oto
44 → 43	**Event** ep moved L relative to h, mo, sb, **Event** mff	Twins (ot, tbr) , **Event** sb moved E relative to cfr,	

	moved E relative to le, olf, **Event** pfb moved L relative to h, sb	mo	
46 → 44	Twins (ep, mff) (kv, fs)	Twins (tl, olf)	
46 → 45	Twins (ep, pfb) (le, tl) (tb, fs), **Event** sb moved L relative to cfr, mo	Twins (ep, pfb) (oto, bc) (op, fs)	Twins (ep, pfb)
48 → 46	Twins (op, fs)	Twins (bc, pfb)	
48 → 47	Twins (ep, bc) (mff, tbr) (sb, h) (tl, ot)	Twins (ep, bc) (sb, h) (tl, ot)	Twins (ep, bc) (sb, h) (tl, ot)
49 → 48	Twins (bc, pfb) , **Event** cfr moved L relative to mo, sb, **Event** tbr moved L relative to kv, ot, tb	Twins (tl, hb) , **Event** tbr moved L relative to kv, tb	**Event** tbr moved L relative to kv, tb
50 → 49	Twins (oto, pfb) (op, tbr), **Event** h moved E relative to cfr, mo, **Event** mff moved E relative to hb, ot, **Event** tl moved E relative to hb, le	Twins (mff, hb) (oto, pfb) (op, tbr) , **Event** h moved E relative to cfr, mo	Twins (oto, pfb) (op, tbr), **Event** h moved E relative to cfr, mo
52 → 50	Twins (oto, bc) (op, fs), **Event** mo moved L relative to cfr, h, sb	Twins (h, mo)	

52 → 51	Twins (cfr, sb) (pfb, ep), **Event** bc moved E relative to mff, oto, **Event** kv moved E relative to op, tbr, **Event** tb moved L relative to fs, le, mo, to, op	Twins (pfb, ep) , **Event** bc moved E relative to mff, oto, **Event** op moved L relative to fs, kv	Twins (pfb, ep) , **Event** bc moved E relative to mff, oto
54 → 53	Twins (mo, cfr) (op, fs)	Twins (op, fs)	Twins (op, fs)
55 → 54	**Event** mff moved L relative to bc, oto, **Event** oto moved L relative to ep, olf, **Event** ot moved L relative to le, mff	Twins (bc, mff)	
56 → 55	Twins (ep, bc) (pfb, h) (sb, cfr), **Event** mff moved L relative to olf, oto, **Event** tb moved E relative to fs, op	Twins (oto, mff) (tl, ot), **Event** tb moved E relative to op, tbr	**Event** tb moved E relative to op, tbr
58 → 56		Twins (cfr, mo) (fs, op) (le, olf) (oto, pfb)	
58 → 57	**Event** mff moved E relative to kv, le, ot, oto moved	Twins (h, bc) (tl, ot), **Event** tbr moved L relative	

	E relative to hb, le, olf, ot, tbr, tl, **Event** op moved E relative to fs, tbr , **Event** pfb moved L relative to cfr, ep	to op, tb	
59 → 58	Twins (tb, tbr), **Event** mo moved L relative to cfr, h , **Event** olf moved L relative to mff, oto, **Event** pfb moved L relative to bc, h, oto, **Event** tl moved E relative to le, ot	Twins (bc, pfb) (fs, kv) (h, mo) , **Event** olf moved L relative to mff, oto, tl	**Event** olf moved L relative to mff, oto, tl
60→ 52		Twins (olf, tl)	
60 → 59	Twins (fs, kv) (h, cfr), **Event** hb moved L relative to oto, tl	Twins (h, cfr) (oto, pfb), **Event** hb moved L relative to mff, tl	Twins (h, cfr), **Event** hb moved L relative to oto, tl
61→ 60	Twins (bc, ep) (fs, op) (h, sb) (oto, pfb)	Twins (bc, ep) (h, sb)	Twins (bc, ep) (h, sb)
32 → *A.xiloaensis*	Twins (h, bc), **Event** ot moved E relative to fs, op, tb	Twins (h, bc), **Event** ot moved E relative to fs, op, tb	Twins (h, bc), **Event** ot moved E relative to fs, op, tb
32→ *C.dimerus*	Twins (cfr, mo) (ep, pfb)	Twins (cfr, mo), **Event** mff moved L relative to bc, h,	Twins (cfr, mo)

		oto, **Event** tl moved L relative to bc, oto	
33→ *H.piceatus*	**Event** sb moved E relative to bc, cfr, ep, h, mff, oto, pfb	**Event** sb moved E relative to bc, cfr, ep, h, mff, oto, pfb, **Event** tb moved L relative to fs, le, ot, **Event** tbr moved L relative to le, ot , **Event** tl moved L relative to hb, le	**Event** sb moved E relative to bc, cfr, ep, h, mff, oto, pfb
33→ *L.trewavasae*	**Event** fs moved L relative to ot, op, tb, **Event** mff moved L relative to bc, pfb, **Event** olf moved L relative to bc, h, hb, le, mff, pfb, **Event** oto moved E relative to bc, hb, le, tl, **Event** tbr moved E relative to op, tb	**Event** fs moved L relative to ot, op, tb, **Event** mff moved L relative to bc, pfb, **Event** olf moved L relative to bc, h, hb, le, mff, pfb, **Event** oto moved E relative to bc, hb, le, tl, **Event** tbr moved E relative to op, tb	**Event** fs moved L relative to ot, op, tb, **Event** olf moved L relative to bc, h, hb, le, mff, pfb, **Event** oto moved E relative to bc, hb, le, tl, **Event** tbr moved E relative to op, tb
34→ *O.niloticus*	Twins (fs, op) (mo, h), **Event** mff moved E relative to bc, oto, **Event** pfb moved E relative to bc,	Twins (mo, h), **Event** fs moved E relative to op, tb, **Event** mff moved E relative to bc, oto, **Event** pfb	Twins (mo, h), **Event** mff moved E relative to bc, oto , **Event** pfb moved E relative to bc, ep, oto

	ep, oto	moved E relative to bc, ep, oto	
36→ *F.heteroclitus*	**Event** olf moved E relative to bc, hb, le, pfb	**Event** olf moved E relative to hb, le, tl	**Event** olf moved E relative to hb, le
36→ *A.xenia*	**Event** oto moved E relative to bc, hb, le	Twins (mff, ep), **Event** olf moved L relative to bc, hb, pfb, **Event** oto moved E relative to bc, hb, le	**Event** oto moved E relative to bc, hb, le
37→ *X.maculatus*	Twins (ot, tbr), **Event** pfb moved E relative to bc, hb, le, olf, **Event** tl moved E relative to bc, hb, le	**Event** tb moved L relative to fs, op, tbr	
38→ *A.myersi*	Twins (sb, mff), **Event** op moved L relative to fs, tb, **Event** tl moved L relative to ep, pfb	Twins (hb, le) (sb, mff), **Event** op moved L relative to fs, kv, **Event** tl moved L relative to bc, ep, oto	Twins (sb, mff) , **Event** op moved L relative to fs, tb , **Event** tl moved L relative to ep
39→ *O.javanicus*	**Event** olf moved E relative to bc, hb, pfb	Twins (cfr, mo) , **Event** tl moved L relative to olf, pfb	
39→ *O.latipes*	Twins (mo, cfr), **Event** olf moved L relative to bc, ep, oto, pfb ,	**Event** olf moved L relative to bc, ep, hb, mff, oto, pfb, **Event** ot	**Event** olf moved L relative to bc, ep, hb, mff, oto, pfb, **Event** ot moved E

	Event ot moved E relative to fs, tbr	moved E relative to fs, tbr	relative to fs, tbr
41→ *M.splendida*	**Event** ep moved E relative to bc, le, oto, pfb, tl, **Event** mff moved L relative to mo, sb, **Event** ot moved L relative to le, tl	**Event** bc moved E relative to hb, oto, **Event** ep moved E relative to bc, hb, le, oto, pfb, tl, **Event** mff moved L relative to mo, sb	**Event** ep moved E relative to bc, hb, le, oto, pfb, tl, **Event** mff moved L relative to mo, sb
43→ *C.striatus*	Twins (sb, mo), **Event** kv moved L relative to le, ot, tbr	**Event** bc moved E relative to hb, oto, **Event** h moved E relative to oto, pfb, **Event** kv moved L relative to fs, ot, tbr, tl, **Event** mff moved E relative to le, tl, **Event** sb moved E relative to mo, pfb	**Event** kv moved L relative to le, ot, tbr
43→ *A.testudineus*	Twins (kv, op), **Event** fs moved L relative to ot, tbr	**Event** ep moved L relative to h, mo, sb, **Event** fs moved L relative to ot, op, tbr	**Event** fs moved L relative to ot, tbr
45→ *G.aculeatus*	Twins (ep, bc) (mo, h), **Event** kv moved L relative to fs, ot, **Event** oto moved E relative	Twins (ep, bc) (mo, h), **Event** kv moved L relative to fs, ot, **Event** le moved E relative	Twins (ep, bc) (mo, h), **Event** kv moved L relative to fs, ot, **Event** oto moved E relative

	to olf, hb, **Event** tbr moved E relative to fs, op	to olf, ot, **Event** oto moved E relative to hb, olf, **Event** tbr moved E relative to fs, op	to hb, olf, **Event** tbr moved E relative to fs, op
45→ *S.vitreum*	**Event** cfr moved E relative to h, mo, sb, **Event** olf moved E relative to hb, le, ot, tbr	Twins (tb, fs), **Event** cfr moved E relative to h, mo, sb, **Event** olf moved E relative to hb, le, ot, **Event** tl moved L relative to le, mff	**Event** cfr moved E relative to h, mo, sb, **Event** olf moved E relative to hb, le, ot, tbr
46→ *G.niger*	**Event** bc moved L relative to ep, pfb, **Event** hb moved L relative to ep, oto, pfb , **Event** ot moved L relative to le, tl, **Event** tb moved L relative to kv, op, **Event** tbr moved L relative to le, ot, tl	**Event** bc moved L relative to ep, oto, pfb, **Event** hb moved L relative to eo, oto, pfb, **Event** ot moved L relative to le, mff, tl. **Event** sb moved E relative to cfr, mo, **Event** tb moved L relative to kv, op, **Event** tbr moved L relative to le, mff, ot, tl	**Event** bc moved L relative to ep, pfb , **Event** hb moved L relative to ep, oto, pfb , **Event** ot moved L relative to le, tl, **Event** tb moved L relative to kv, op, **Event** tbr moved L relative to le, ot, tl
47→ *L.petersii*	Twins (tbr, ot) (tl, le), **Event** mo moved E relative to h, sb	Twins (mo, h) (tl, le)	Twins (tl, le)

49→ *G.morhua*	Twins (pfb, ep) (sb, mo), **Event** fs moved E relative to es, kv, op, **Event** tb moved L relative to kv, op	Twins (pfb, ep), **Event** fs moved E relative to es, kv, op, **Event** mff moved E relative to le, olf, ot, **Event** mo moved L relative to cfr, sb, **Event** olf moved E relative to hb, le, **Event** ot moved E relative to bc, hb, le, olf, **Event** tb moved L relative to kv, op	Twins (pfb, ep), **Event** fs moved E relative to es, kv, op, **Event** tb moved L relative to kv, op
50→ *G.maculatus*	**Event** ep moved E relative to mff, oto, pfb, tl, **Event** fs moved L relative to le, op, tb, tbr, **Event** hb moved E relative to le, olf, tl, **Event** kv moved L relative to le, tbr, **Event** ot moved L relative to le, olf, **Event** pfb moved E relative to mff, oto	Twins (cfr, mo) , **Event** ep moved E relative to oto, pfb, tl, **Event** fs moved L relative to op, tb, **Event** hb moved E relative to le, olf, tl, **Event** le moved E relative to olf, tl	**Event** ep moved E relative to mff, oto, pfb, tl, **Event** fs moved L relative to le, op, tb, tbr, **Event** hb moved E relative to le, olf, tl
51→ *O.mykiss*	**Event** olf moved E relative to hb, le	Twins (ep, oto) (kv, tbr), **Event**	**Event** olf moved E relative to hb, le,

	, **Event** ot moved L relative to bc, hb, le, mff	olf moved E relative to hb, le, **Event** ot moved L relative to bc, hb, mff, tl	**Event** ot moved L relative to bc, hb, le, mff
51→ *S.salar*	**Event** mo moved E relative to bc, ep, hb, mff, oto, **Event** olf moved L relative to hb, mff	Twins (cfr, sb), **Event** mo moved E relative to bc, ep, hb, mff, oto, **Event** olf moved L relative to bc, hb, le, mff, **Event** pfb moved L relative to bc, oto, **Event** tb moved L relative to fs, kv, le, ot, op	**Event** mo moved E relative to bc, ep, hb, mff, oto, **Event** olf moved L relative to hb, mff
53→ *C.auratus*	Twins (mo, h) (op, tbr), **Event** mff moved E relative to le, pfb, tl, **Event** olf moved L relative to hb, pfb	Twins (mo, h) (op, tbr) (tb, fs), **Event** hb moved L relative to ep, pfb, **Event** mff moved E relative to le, tl, **Event** olf moved L relative to ep, hb, pfb	Twins (mo, h) (op, tbr), **Event** mff moved E relative to le, pfb, tl, **Event** olf moved L relative to hb, pfb
53→ *C.carpio*	Twins (sb, mo), **Event** mff moved L relative to ep, h, hb, olf, pfb, **Event** ot moved E relative to kv, le, tl	**Event** ep moved E relative to bc, pfb, **Event** mff moved L relative to h, hb, oto, pfb, **Event** ot moved E	**Event** mff moved L relative to h, hb, oto, pfb, **Event** ot moved E relative to kv, tl, **Event** tbr moved E relative

	, **Event** pfb moved L relative to ep, hb, **Event** tbr moved E relative to kv, op	relative to kv, tl, **Event** tbr moved E relative to kv, op	to kv, op
54→ *B.gonionotus*	Twins (fs, tb), **Event** cfr moved E relative to h, mo, sb, **Event** ep moved L relative to h, mo, pfb, **Event** ot moved L relative to hb, mff, pfb, tl	**Event** cfr moved E relative to h, mo, sb, **Event** ep moved L relative to h, mo, **Event** ot moved L relative to hb, mff, tl	**Event** cfr moved E relative to h,mo, sb , **Event** ep moved L relative to h, mo, **Event** ot moved L relative to hb, mff, tl
55→ *D.rerio*	**Event** bc moved L relative to oto, pfb, **Event** hb moved L relative to mff, olf, oto, **Event** sb moved E relative to h, mo, **Event** tbr moved L relative to ot, op	Twins (tb, fs), **Event** bc moved L relative to oto, pfb, **Event** hb moved L relative to mff, olf, oto, **Event** sb moved E relative to cfr, h, mo , **Event** tbr moved L relative to to, op	**Event** bc moved L relative to oto, pfb, **Event** hb moved L relative to mff, olf, oto, **Event** sb moved E relative to h, mo, **Event** tbr moved L relative to ot, op
56→ *C.commersoni*	Twins (h, ep), **Event** cfr moved E relative to mo, sb, **Event** olf moved L relative to bc, hb	Twins (fs, tb), **Event** cfr moved E relative to mo, sb, **Event** h moved E relative to ep, pfb, **Event** kv moved L	**Event** cfr moved E relative to mo, sb, **Event** olf moved L relative to bc, hb

		relative to ot, tbr, **Event** olf moved L relative to bc, hb, mff	
57→ *H.bidorsalis*	Twins (mo, cfr), **Event** le moved L relative to bc, hb, olf, tl, **Event** olf moved L relative to bc, hb, **Event** tbr moved L relative to hb, kv, ot, tl	**Event** le moved L relative to bc, hb, tl, **Event** olf moved L relative to bc, hb, **Event** oto moved E relative to ot, tl, **Event** tbr moved L relative to hb, kv, ot, tl	**Event** le moved L relative to bc, hb, olf, tl, **Event** olf moved L relative to bc, hb, **Event** tbr moved L relative to hb, kv, ot, tl
57→ *H.fossilis*	**Event** kv moved L relative to le, tbr, tl, **Event** ot moved L relative to hb, le, olf, tl	**Event** kv moved L relative to tbr, tl, **Event** mff moved E relative to hb, le, tl, **Event** ot moved L relative to hb, olf, tl	**Event** kv moved L relative to le, tbr, tl, **Event** ot moved L relative to hb, le, olf, tl
59→ *A.sapidissima*	**Event** tb moved L relative to fs, ot, op	**Event** hb moved L relative to bc, mff, olf, oto, **Event** ot moved L relative to le, olf, **Event** op moved L relative to fs, tbr, **Event** tb moved L relative to fs, le, olf, ot,	**Event** tb moved L relative to fs, ot, op

		op, tbr, **Event** tl moved L relative to le, mff, olf	
61→ *A.calva*	Twins (hb,le), **Event** oto moved L relative to bc, ep, mo, **Event** tbr moved L relative to op, tb	Twins (hb, le), **Event** oto moved L relative to bc, ep, mo, **Event** tbr moved L relative to op, tb	Twins (hb,le), **Event** oto moved L relative to bc, ep, mo, **Event** tbr moved L relative to op, tb

Table 4. List of heterochronic shifts detected by Parsimov methods

The detected heterochronic shifts in each branch were listed. Each shift was categorized relative movement (E = earlier; L = later) and rank change only between two events (Twins). The node numbers were shown in Figure 1.

Branch name	Branch length (genertion/Mya)	Reference
Adinia xenica	70.00	Cunningham and Balon 1986
Alosa sapidissima	61.63	Scott and Crossman 1998
Amphilophus xiloaensis	10.75	Kratochwil et al., 2015
Anabas testudineus	68.60	Singh et al., 2012
Austrofundulus myersi	106.05	Wourms JP 1998
Barbodes gonionotus	204.60	Basak et al.,2014
Carassius auratus	40.80	Massimoet al., 2010
Catostomus commersoni	27.40	Chen and Harvey 1994
Channa striatus	137.20	Yaakov and Ali 1992
Cichlasoma dimerus	16.13	Pandolfi et al., 2009
Cyprinus carpio	24.48	Snyder et al., 2004
Danio rerio	175.20	Engeszer et al., 2007
Fundulus heteroclitus	23.33	Shimizuet al., 2008
Gadus morhua	73.00	Kolstad et al., 2006
Galaxias maculatus	202.00	Burnet 1965
Gasterosteus aculeatus	36.40	Bell and Foster 1994
Gobius niger	18.60	Boban et al., 2013
Haplochromis piceatus	2.67	Jong et al., 2009
Heterobranchus bidorsalis	142.70	Yalçin et al., 2001
Heteropneustes fossilis	142.70	Hossain et al., 2015
Labeotropheus trewavasae	2.67	Balon 1977
Leucopsarion petersii	37.20	Takegaki et al., 2013
Melanotaenia splendida	123.45	Humphreyet al., 2003
Oncorhynchus mykiss	16.80	Behnke 1992
Oreochromis niloticus	18.20	Fujimura and Olada 2007
Oryzias javanicus	95.25	Kakuno et al., 2001
Oryzias latipes	152.40	Christian et al., 2012
Salmo salar	11.20	Gjerde 1984
Stizostedion vitreum	10.92	Colby et al., 1979
Xiphophorus maculatus	76.63	Tavolga and Rugh 1947

Table 5. List of branch length scaled by generation number and references

Taxon	Order	Hatch time(hpf)	Number of events after hatch
Amia calva	Amiiformes	**264**	**0**
Alosa sapidissima	Clupeiformes	**160**	**1**
Catostomus commersoni	Cypryniformes	456–480	4
Danio rerio	Cypryniformes	48	2
Cyprinus carpio	Cypryniformes	78	5
Carassius auratus	Cypryniformes	58	2
Barbodes gonionotus	Cypryniformes	13.4	4
Heteropneustes fossilis	Siluriformes	23–24	5
Heterobranchus bidorsalis	Siluriformes	21	7
Oncorhynchus mykiss	Salmoniformes	672	0
Salmo salar	Salmoniformes	280–285	1
Galaxias maculatus	Galaxiiformes	240	1
Gadus morhua	Gadiformes	256	4
Gobius niger	Gobiiformes	144	3
Leucopsarion petersii	Gobiiformes	312	0
Gasterosteus aculeatus	Perchiformes	192	3
Stizostedion vitreum	Perchiformes	162.5	2
Channa striatus	Anabantiformes	23.5–24	5
Anabas testudineus	Anabantiformes	20	5
Amphilophus xiloaensis	Cichliformes	50–60	7
Cichlasoma dimerus	Cichliformes	54	6
Oreochromis niloticus	Cichliformes	90	2
Labeotropheus trewavasae	Cichliformes	153	4
Haplochromis piceatus	Cichliformes	120–124	3
Melanotaenia splendida	Atheriniformes	105	2
Adinia xenica	Cyprinodontiformes	216–234	0
Fundulus heteroclitus	Cyprinodontiformes	228	0
Xiphophorus maculatus	Cyprinodontiformes	192	1
Austrofundulus myersi	Cyprinodontiformes	936	0
Oryzias latipes	Beloniformes	216	0
Oryzias javanicus	Beloniformes	216–240	0

Table 6. List of duration of embryonic period and the number of events occurred after hatch event