1 Title: **Frequent Non-random Shifts in the Temporal Sequence of Developmental Landmark**

2 **Events during Teleost Evolutionary Diversification**

3

4 Running head: **Evolution of teleost developmental sequence**

5

6 **Authors**

7 Fumihiro Ito^{1,2,*}, Tomotaka Matsumoto^{2,3}, Tatsumi Hirata^{2,4}

8 ¹Mammalian Genetics Laboratory, Genetic Strains Research Center, National Institute of
9 Genetics, Mishima 411-8540, Shizuoka, Japan

10 ²Department of Genetics, SOKENDAI (The Graduate University for Advanced Studies),
11 Mishima 411-8540, Shizuoka, Japan

12 ³Division of Evolutionary Genetics, Department of Population Genetics, National Institute of
13 Genetics, Yata 1111, Mishima, Shizuoka 411-8540, Japan.

14 ⁴Division of Brain Function, National Institute of Genetics, Yata 1111, Mishima 411-8540,
15 Shizuoka, Japan.

16

17 *Correspondence author

18

19 **Abstract**

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

36

37 **Key Words**

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

39

40

41 **1. Introduction**

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

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

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

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

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

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

97

98 **2. Materials and Methods**

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

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

107

108 **2.2 Data sampling**

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

122

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

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

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

130

131 **2.4 Reconstruction of ancestral developmental sequences**

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

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

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

150

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

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

159

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

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

167

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

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

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

177

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

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

183

184 **3. Results**

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

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

194 species covered in this study were basically freshwater teleosts; however, several anadromous
 195 species were also included such as *Gasterosteus aculeatus*, which develops in freshwater but
 196 alternates between the sea and freshwater as an adult.

197

198 **3.2 Comparison of temporal orders of developmental events among teleost species**

199 We selected 19 developmental events that appeared consistently as landmarks in the
 200 developmental staging of many teleost species (Figure 1). To gain an overall view of
 201 developmental sequences involved in the formation of entire body, we intentionally included
 202 events that belong to substantially different biological systems and contexts such as those
 203 originating from different germ layers or giving rise to different cell types or separate body parts.
 204 Additionally, the list also included a small number of interrelated events such as formation of the
 205 optic vesicles/placodes/primordia (op), lenses or lens placodes (le), and eye pigmentation (ep).
 206 We gathered information on these 19 events from articles reporting the development of 31
 207 actinopterygian species, and ranked the orders of individual events in the temporal sequence for
 208 each species (Figure 1).

209 We first compared rank orders of individual events among 30 in-group teleost species.
 210 To minimize effects of simultaneous occurrence of events and missing data, the raw ranks (Figure
 211 1) were rescaled to the normalized ranks that fit within the same range, from 0 to 1, in all the
 212 teleost species (see Methods). Figure 3a shows the distribution of the normalized ranks for
 213 individual developmental events, which were horizontally arranged according to the average
 214 values. Interestingly, the ranges of variation in the ranks differed widely, depending on the event.
 215 One extreme case was embryonic shield (es), which always appeared first and with no variation in

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

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

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

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

243

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

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

low-profile events such as blood circulation (bc), we do not consider the weak upward trend as a continual ontogenic pattern formed by temporally clustered events.

We also examined evolutionary rank changes using a different index. Based on continuous analysis (Germain & Laurin, 2009), the evolutionary rate of rank changes can be obtained for each event (Figure 4b). This index shows rank changeability per million years and therefore differs slightly from the one per branch used in the previous analysis. The calculation also involved the set of different ancestral sequences estimated by continuous analysis. Nevertheless, this new index was basically similar to the previous analysis, with the olfactory vesicles/pits/primordia (olf) exhibiting high rank changeability. In the ontogenic sequence, this high score event was like an independent peak with no indication of a global ontogenic pattern. These and previous results further suggest that the ranks of olfactory vesicles/pits/primordia (olf) have changed more frequently during evolution than the other events.

3.4 Event exchange rates in developmental sequences

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

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

3.5 Distribution of heterochronic shifts across the teleost phylogenetic tree

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

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

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

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

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

334

335 **4. Discussion**

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

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

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

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

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

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

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

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

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

Acknowledgements

We thank Dr. Toshihiko Shiroishi, Dr. Yasushi Hiromi, Dr. Naoki Irie, Dr. Takanori Amano, Dr. Yuuta Moriyama, and Dr. Kousuke Mouri for comments that helped to greatly improve the manuscript. We also thank Dr. Erin E. Maxwell, Dr. Luke B. Harrison, and Dr. Atsushi Kawakita for their advice on parsimony analyses. T.H. acknowledges the funding support from

414 MEXT/JSPS KAKENHI Grants (17H05776, 17H05587, 16H04659). This work was supported

415 in part by SOKENDAI (The Graduate University for Advanced Studies).

416

417

418

419 **Reference**

420 Arakawa, T., Kanno, Y., Akiyama, N., Kitano, T., Nakatsuji, N., & Nakatsuji, T. (1999). Stages
421 of embryonic development of the ice goby (shiro-uo), *Leucopsarion petersii*. *Zoological Science*,
422 16, 761-773. doi.org/10.2108/zsj.16.761

423
424 Armstrong, P. B., & Child J. S. (1965). Stages in normal development of *Fundulus heteroclitus*.
425 *The Biological Bulletin*, 128, 143-168. doi:10.2307/1539545

426
427 Ballard, W. W. (1969). Normal embryonic stages of *Gobius niger* jozo. *Pubbl Staz Zool Napoli*,
428 37, 1-17.

429
430 Ballard, W. W. (1973). Normal embryonic stages for salmonid fishes, based on *Salmo gairdneri*
431 Richardson and *Salvelinus fontinalis* (Mitchill). *Journal of Experimental Zoology Part A:*
432 *Ecological and Integrative Physiology*, 184, 7-26. doi: 10.1002/jez.1401840103

433
434 Ballard, W. W. (1986). Morphogenetic movements and a provisional fate map of development
435 in the holostean fish, *Amia calva*. *Journal of Experimental Zoology Part A: Ecological and*
436 *Integrative Physiology*, 238, 355-372. doi:10.1002/jez.1402380308

437
438 Balon, E. K. (1977). Early ontogeny of *Labeotropheus* Ahl, 1927 (Mbuna, Cichlidae, Lake
439 Malawi), with a discussion on advanced protective styles in fish reproduction and development.
440 *Environmental Biology of Fishes*, 2, 147-176.

441 Basak, S. K., Basak, B., Gupta, N., Haque, M. M., & Amin, R. (2014). Embryonic and larval
442 development of silver barb (*Barodes gonionotus*) in a mobile hatchery under laboratory
443 Condition. *European Scientific Journal, Special ed*, 3, 258-270.
444
445 Benzie, V. (1968). Stages in the normal development of *Galaxias maculatus attenuatus* (Jenyns).
446 *New Zealand Journal of Marine and Freshwater Research*, 2, 606-627.
447
448 Bininda-Emonds, O. R., Jeffery, J. E., & Richardson, M. K. (2003). Inverting the hourglass:
449 quantitative evidence against the phylotypic stage in vertebrate development. *Proceedings of the*
450 *Royal Society B: Biological Sciences*, 270, 341–346. doi: 10.1098/rspb.2002.2242
451
452 Carril, J., & Tambussi, C. P. (2017). Skeletogenesis of *Myiopsitta monachus* (Psittaciformes)
453 and sequence heterochronies in Aves. *Evolution & Development*, 19, 17-28.
454 doi:10.1111/ede.12211
455
456 Cunningham, J. E. R., & Balon, E. K. (1985). Early ontogeny of *Adinia xenica* (Pisces,
457 Cyprinodontiformes): 1. The development of embryos in hiding. *Environmental Biology of*
458 *Fishes*, 14, 115-166.
459
460 de Jong, I. M., Colbert, M. W., Witte, F., & Richardson, M. K. (2009). Polymorphism in
461 developmental timing: intraspecific heterochrony in a Lake Victoria cichlid. *Evolution &*
462 *Development*, 11, 625-635. doi:10.1111/j.1525-142X.2009.00370.x

463

464 de Jong, I. M., Witte, F., & Richardson, M. K. (2009). Developmental stages until hatching of
465 the Lake Victoria cichlid *Haplochromis piceatus* (Teleostei: Cichlidae). *Journal of Morphology*,
466 270, 519-535. doi:10.1002/jmor.10716

467

468 Domazet-Lošo, T., & Tautz, D. (2010). A phylogenetically based transcriptome age
469 index mirrors ontogenetic divergence patterns. *Nature* 468, 815–818, doi: 10.1038/nature09632.

470

471 Duboule, D. (1994). Temporal colinearity and the phylotypic progression: a basis for the
472 stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony.
473 *Development (suppl.)*, 135-142.

474

475 Felsenstein, J. (1978). Cases in which Parsimony or Compatibility Methods will be Positively
476 Misleading. *Systematic Biology*, 27 (4), 401–410. doi.org/10.1093/sysbio/27.4.401.

477

478 Friedman, M., Keck, B. P., Dornburg, A., Eytan, R. I., Martin, C. H., Hulsey, C. D., Wainwright,
479 P. C., & Near, T. J. (2013). Molecular and fossil evidence place the origin of cichlid fishes long
480 after Gondwanan rifting. *Proceedings of the Royal Society B: Biological Sciences*, 280,
481 20131733. doi:10.1098/rspb.2013.1733

482

483

484 Fujimura, K., & Okada, N. (2007). Development of the embryo, larva and early juvenile of Nile
485 tilapia *Oreochromis niloticus* (Pisces: Cichlidae). Developmental staging system. *Development*
486 *Growth & Differentiation*, 49, 301-324. doi:10.1111/j.1440-169X.2007.00926.x
487
488 Germain, D., & Laurin, M. (2009). Evolution of ossification sequences in salamanders and
489 urodele origins assessed through event-pairing and new methods. *Evolution & Development*, 11,
490 170-190. doi:10.1111/j.1525-142X.2009.00318.x
491
492 Gorodilov, Y. N. (1996). Description of the early ontogeny of the Atlantic salmon, *Salmo salar*,
493 with a novel system of interval (state) identification. *Environmental Biology of Fishes*.
494 47(2) :109–127. doi: 10.1007/BF00005034
495
496 Haeckel, E. (1874). Anthropogenie oder Entwicklungsgeschichte des Menschen. *Engelmann*,
497 *Leipzig*.
498
499 Hall, T. E., Smith, P., & Johnston, I. A. (2004). Stages of embryonic development in the
500 Atlantic Cod *Gadus morhua*. *Journal of Morphology*, 259, 255-270. doi: 10.1002/jmor.10222
501
502 Harrison, L. B., & Larsson, H. C. (2008). Estimating evolution of temporal sequence changes: a
503 practical approach to inferring ancestral developmental sequences and sequence heterochrony.
504 *Systematic Biology*, 57, 378-387. doi:10.1080/10635150802164421
505

506 Hautier, L., Weisbecker, V., Goswami, A., Knight, F., Kardjilov, N., & Asher, R. J. (2011).
507 Skeletal ossification and sequence heterochrony in xenarthran evolution. *Evolution &*
508 *Development*, 13, 460-476. doi:10.1111/j.1525-142X.2011.00503.x
509
510 Hautier, L., Bennett, N. C., Viljoen, H., Howard, L., Milinkovitch, M. C., Athanasia, C. T.,
511 Goswami, A., & Asher, R. J. (2013). Patterns of ossification in southern versus northern
512 placental mammals. *Evolution* 67:1994–2010. doi: 10.1111/evo.12071
513
514 Hedges, S. B. & Kumar, S. (2009). The timetree of life. New York, NY: Oxford University
515 Press.
516
517 Humphrey, C., Klumpp, D. W., & Pearson, R. (2003). Early development and growth of the
518 east rainbowfish, *Melanotaenia splendida splendida*. *Marine and Freshwater Research*, 54,
519 17-25. doi: org/10.1071/MF02037
520
521 Irie, N., & Kuratani, S. (2011). Comparative transcriptome analysis reveals vertebrate
522 phylotypic period during organogenesis. *Nature Communications*, 2, 248.
523 doi:10.1038/ncomms1248
524
525 Iwamatsu, T. (2004). Stages of normal development in the medaka *Oryzias latipes*. *Mechanisms*
526 *of Development*, 121, 605-618. doi:10.1016/j.mod.2004.03.012
527

528 Iwamatsu, T., & Hirata, K. (1984). Normal course of development of the Java medaka, *Oryzias*
529 *javanicus*. *Bulletin of Aichi University of Education* (Natural Science), 33, 87-109.

530

531 Jeffery, J. E., Bininda-Emonds, O. R., Coates, M. I., & Richardson, M. K. (2002a). Analyzing
532 evolutionary patterns in amniote embryonic development. *Evolution & Development*, 4, 292-302.
533 doi: 10.1046/j.1525-142X.2002.02018.x

534

535 Jeffery, J. E., Richardson, M. K., Coates, M. I., & Bininda-Emonds, O. R. (2002b). Analyzing
536 developmental sequences within a phylogenetic framework. *Systematic Biology*, 51, 478-491.
537 doi:10.1080/10635150290069904

538

539 Jeffery, J. E., Bininda-Emonds, O. R., Coates, M. I., & Richardson, M. K. (2005). A new
540 technique for identifying sequence heterochrony. *Systematic Biology*, 54, 230-240.
541 doi:10.1080/10635150590923227

542

543 Kalinka, A. T., Varga, K. M., Gerrard, D. T., Preibisch, S., Corcoran, D. L., Jarrells, J., Ohler,
544 U., Bergmen, C.M., & Tomancak, P. (2010). Gene expression divergence recapitulates the
545 developmental hourglass model. *Nature*, 468, 811-814. doi:10.1038/nature09634

546

547 Kawanishi, T., Kaneko, T., Moriyama, Y., Kinoshita, M., Yokoi, H., Suzuki, T., Shimada, A.,
548 & Takeda, H. (2013). Modular development of the teleost trunk along the dorsoventral axis and

549 *zic1/zic4* as selector genes in the dorsal module. *Development*, 140, 1486-1496.

550 doi:10.1242/dev.088567

551

552 Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T.F. (1995). Stages of

553 embryonic development of the zebrafish. *Developmental Dynamics*, 203, 253-310.

554 doi:10.1002/aja.1002030302

555

556 Klingenberg, C. P., Badyaev, A. V., Sowry, S. M., & Beckwith, N. J. (2001). Inferring

557 developmental modularity from morphological integration: analysis of individual variation and

558 asymmetry in bumblebee wings. *American Naturalist*, 157, 11-23. doi:10.1086/317002

559

560 Kratochwil, C. F., Sefton, M. M., & Meyer, A. (2015). Embryonic and larval development in

561 the Midas cichlid fish species flock (*Amphilophus spp.*): a new evo-devo model for the

562 investigation of adaptive novelties and species differences. *BMC Developmental Biology*, 15, 12.

563 doi:10.1186/s12861-015-0061-1

564

565 Laurin, M. (2014). Assessment of modularity in the urodele skull: an exploratory analysis using

566 ossification sequence data. *Journal of Experimental Zoology Part B: Molecular and*

567 *Developmental Evolution*, 322, 567-585. doi:10.1002/jez.b.22575

568

569 Long, W. L., & Ballard, W. W., (1976). Normal embryonic stages of the white sucker,

570 *Catostomus commersoni*. *Copeia*, 1976, 342-351. doi. 10.2307/1443957

571

572 Mabee, P. M., Olmstead, K. L., & Cubbage, C.C. (2000). An experimental study of intraspecific
573 variation, developmental timing, and heterochrony in fishes. *Evolution*, 54, 2091-2106

574

575 Maddison, W. P. (1991). Squared-change parsimony reconstructions of ancestral
576 states for continuous—valued characters on a phylogenetic tree. *Systematic Zoology*.
577 40:304–314. doi: 10.2307/2992324

578

579 Maddison, W. P., & D. R. Maddison. (2002). Mesquite: a modular system for evolutionary
580 analysis. Version 3.3.1.

581

582 Marimuthu, K., & Haniffa, M. A. (2007). Embryonic and larval development of the striped
583 snakehead *Channa striatus*. *Taiwania*, 52, 84-92. doi:10.6165/tai

584

585 Maxwell, E. E., Harrison, L. B., & Larsson, H. C. (2010). Assessing the phylogenetic utility of
586 sequence heterochrony: evolution of avian ossification sequences as a case study. *Zoology*
587 (*Jena*), 113, 57-66. doi:10.1016/j.zool.2009.06.002

588

589 McElman, J. F., & Balon, E. K. (1979). Early ontogeny of walleye, *Stizostedion vitreum*, with
590 steps of saltatory development. *Environmental Biology of Fishes*, 4, 309-348.

591

592 Meijide, F. J., & Guerrero, G. A. (2000). Embryonic and larval development of a
593 substrate-brooding cichlid *Cichlasoma dimerus* (Heckel, 1840) under laboratory conditions.
594 *Journal of Zoology*, 252, 481-493. doi: 10.1111/j.1469-7998.2000.tb01231.x
595
596 Midford, P., Garland, T. J., & Maddison. W. P. (2003). PDAP package for Mesquite.
597
598 Mitgutsch, C., Wimmer, C., Sanchez-Villagra, M. R., Hahnloser, R., & Schneider, R. A. (2011).
599 Timing of ossification in duck, quail, and zebra finch: intraespecific variation, heterochronies,
600 and life story evolution. *Zoological. Science*, 28: 491–500. doi: 10.2108/zsj.28.491.
601
602 Near, T. J., Eytan, R. I., Dornburg, A., Kuhn, K. L., Moore, J. A., Davis, M. P., Wainwright, P.
603 C.. Friedman, M., & Smith, W. L. (2012). Resolution of ray-finned fish phylogeny and timing
604 of diversification. *Proceedings of the National Academy of Sciences of the United States of*
605 *America*, 109, 13698-13703. doi:10.1073/pnas.1206625109
606
607 Near, T. J., Dornburg, A., Eytan, R. I., Keck, B. P., Smith, W. L., Kuhn, K. L., Moore, J. A.,
608 Price, S. A., Burbrink, F. T., Friedman, M., & Wainwright, P. C. (2013). Phylogeny and tempo
609 of diversification in the superradiation of spiny-rayed fishes. *Proceedings of the National*
610 *Academy of Sciences of the United States of America*, 110, 12738-12743.
611 doi:10.1073/pnas.1304661110
612

613 Nelson, J. S., Grande, T. C., & Wilson, M. V. H. (2016). Fishes of the world (5th ed). New
614 York: Wiley.

615

616 Nunn, C. L., & Smith, K. K. (1998). Statistical analyses of developmental sequences: the
617 craniofacial region in marsupial and placental mammals. *American Naturalist*, 152, 82-101.
618 doi:10.1086/286151

619

620 Olaniyi, W. A., & Omitogun, O. G. (2014). Embryonic and larval developmental stages of
621 African giant catfish *Heterobranchus bidorsalis* (Geoffroy Saint Hilaire, 1809) (Teleostei,
622 Clariidae). *Springerplus*, 3, 677. doi:10.1186/2193-1801-3-677

623

624 Pelluet, D. (1944) Criteria for the recognition of development stages in the salmon (*Salmo*
625 *salar*). *Journal of Morphology*, 74, 395-407. doi: 10.1002/jmor.1050740305

626

627 Perez, G. A., Rican, O., Orti, G., Bermingham, E., Doadrio, I., & Zardoya, R. (2007).
628 Phylogeny and biogeography of 91 species of heroine cichlids (Teleostei: Cichlidae) based on
629 sequences of the cytochrome b gene. *Molecular Phylogenetics and Evolution*, 43, 91-110.
630 doi:10.1016/j.ympev.2006.08.012

631

632 Poe, S., & Wake, M. H. (2004). Quantitative tests of general models for the evolution of
633 development. *American Naturalist*. 164:415–422.

634

635 Pohl, M., Milvertz, F. C., Meyer, A., & Vences, M. (2015). Multigene phylogeny of
636 cyprinodontiform fishes suggests continental radiations and a rogue taxon position of
637 Pantanodon. *Vertebrate Zoology*, 65, 37-44.
638
639 Puvaneswari, S., Marimuthu, K., Karuppasamy, R., & Haniffa, M. A. (2009). Early embryonic
640 and larval development of Indian catfish, *Heteropneustes fossilis*. *EurAsian Journal of*
641 *BioSciences* 3, 84-96. doi: 10.5053/ejobios.2009.3.0.12
642
643 Raff, R. A. (1996). The shape of life: genes, development, and the evolution of animal form.
644 Chicago: Univ of Chicago Press
645
646 Richardson, M. K. (1999). Vertebrate evolution: the developmental origins of adult variation.
647 *Bioessays* 21: 604–613. doi:
648 10.1002/(SICI)1521-1878(199907)21:7<604::AID-BIES9>3.0.CO;2-U
649
650 Richardson, M. K. (2012). A phylotypic stage for all animals? *Developmental Cell*. 22(5):903–4.
651 doi:10.1016/j.devcel.2012.05.001
652
653 Romer, A. S., & Parsons, T. S. (1986). The vertebrate body (6th ed). Philadelphia, PA:
654 Saunders College Publications.
655

656 Saitoh, K., Sado, T., Doosey, M. H., Bart Jr., H. L., Inoue, J. G., Nishida, M., Mayden, R. L., &
657 Miya, M. (2011). Evidence from mitochondrial genomics supports the lower Mesozoic of South
658 Asia as the time and place of basal divergence of cypriniform fishes (Actinopterygii:
659 Ostariophysi). *Zoological Journal of the Linnean Society*, 161, 633 – 662.
660 doi:10.1111/j.1096-3642.2010.00651.x
661
662 Sanchez-Villagra, M. R., Goswami, A., Weisbecker, V., Mock, O., & Kuratani, S. (2008).
663 Conserved relative timing of cranial ossification patterns in early mammalian evolution.
664 *Evolution & Development*, 10, 519-530. doi:10.1111/j.1525-142X.2008.00267.x
665
666 Schlosser, G. (2008). Development of the retinotectal system in the direct-developing frog
667 *Eleutherodactylus coqui* in comparison with other anurans. *Frontiers in Zoology*, 5, 9.
668 doi:10.1186/1742-9994-5-9
669
670 Schmidt, K., & Starck, J. M. (2010). Developmental plasticity, modularity, and heterochrony
671 during the phylotypic stage of the zebra fish, *Danio rerio*. *Journal of Experimental Zoology Part*
672 *B: Molecular and Developmental Evolution*, 314, 166-178. doi:10.1002/jez.b.21320
673
674 Schoch, R. R. (2006). Skull ontogeny: developmental patterns of fishes conserved across major
675 tetrapod clades. *Evolution & Development*, 8, 524-536. doi:10.1111/j.1525-142X.2006.00125.x
676

677 Shardo, J. D. (1995). Comparative embryology of teleostean fishes. I. Development and staging
678 of the American shad, *Alosa sapidissima* (Wilson, 1811). *Journal of Morphology*, 225, 125-167.
679 doi:10.1002/jmor.1052250202
680
681 Smith, K. K. (2001). Heterochrony revisited: the evolution of developmental sequences.
682 *Zoological Journal of the Linnean Society*. 73: 169–186. doi: 10.1006/bijl.2001.0535
683
684 Smirthwaite, J. J., Rundle, S. D., Bininda-Emonds, O. R., & Spicer J. I. (2007). An integrative
685 approach identifies developmental sequence heterochronies in freshwater basommatophoran
686 snails. *Evolution & Development*, 9, 122-130. doi:10.1111/j.1525-142X.2007.00143.x
687
688 Strauss, R. E. (1990). Heterochronic variation in the developmental timing of cranial
689 ossifications in poeciliid fishes (Cyprinodontiformes). *Evolution*, 44, 1558-1567.
690 doi:10.1111/j.1558-5646.1990.tb03846.x
691
692 Swarup, H. (1958). Stages of development of the stickleback *Gasterosteus aculeatus*. *Journal of*
693 *Embryology and Experimental Morphology*, 6, 373-383.
694
695 Swofford, D. L. (2002). PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods),
696 v. 4beta10. Sunderland, MA: Sinauer.
697

698 Tavalga, W. N., & Rugh, R. (1947). Development of the platyfish, *Platypoecilus maculatus*.
699 *Zoologica; Scientific Contributions of the New York Zoological Society*, 32, 1-15.
700
701 Tsai, H. Y., Chang, M., Liu, S. C., Abe, G., & Ota, K. G. (2013). Embryonic development of
702 goldfish (*Carassius auratus*): a model for the study of evolutionary change in developmental
703 mechanisms by artificial selection. *Developmental Dynamics*, 242, 1262-1283.
704 doi:10.1002/dvdy.24022
705
706 Verma, P. (1970). Normal stages in the development of *Cyprinus carpio* var. communis L. *Acta*
707 *Biologica Academiae Scientiarum Hungaricae*, 21, 207-218.
708
709 Weisbecker, V., & Mitgutsch, M. (2010). A large-scale survey of heterochrony in anuran cranial
710 ossification patterns. *Journal of Zoological Systematics and Evolutionary Research*. 48 (4),
711 332–347. doi: 10.1111/j.1439-0469.2010.00570.x
712
713 Werneburg, I., & Sanchez-Villagra, M. R. (2015). Skeletal heterochrony is associated with the
714 anatomical specializations of snakes among squamate reptiles. *Evolution* 69, 254–263. doi:
715 10.1111/evo.12559.
716
717 Workman, A. D., Charvet, C. J., Clancy, B., Darlington, R. B., & Finlay, B. L. (2013).
718 Modeling transformations of neurodevelopmental sequences across mammalian species.
719 *Journal of Neuroscience*, 33, 7368-7383. doi:10.1523/JNEUROSCI.5746-12.2013

720

721 Wourms, J. P. (1972). Developmental biology of annual fishes. I. Stages in the normal

722 development of *Austrofundulus myersi* Dahl. *Journal of Experimental Zoology Part A:*

723 *Ecological and Integrative Physiology*, 182, 143-167. doi:10.1002/jez.1401820202

724

725 Yang, L., Sado, T., Vincent Hirt, M., Pasco-Viel, E., Arunachalam, M., Li, J., Wang, X.,

726 Freyhof, J., Saitoh, K., Simons, A. M., Miya, M., He, S., & Mayden, R. L. (2015). Phylogeny

727 and polyploidy: resolving the classification of cyprinine fishes (Teleostei: Cypriniformes).

728 *Molecular Phylogenetics and Evolution*, 85, 97-116. doi:10.1016/j.ympev.2015.01.014

729

730 Zalina, I., Saad, C. R., Christianus, A., & Harmin, S. A. (2012). Induced breeding and

731 embryonic development of climbing perch (*Anabas testudineus*, Bloch). *Journal of Fisheries*

732 *and Aquatic Science*, 7, 291-306. doi.org/10.5567/ECOLOGY-IK.2013.8.14

733

734

735 **Author contribution**

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

739

740

741 **Competing interests**

742 The authors have no competing interests.

743

744

745 **Figure Legends**

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

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

755

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

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

762

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

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

770

771

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

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

779 obtained by continuous analysis (Germain & Laurin, 2009). The events are arranged in the same
780 order as in (a).

781

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

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

784 The number shows the percentage of the sequences in which the row event occurred later than

785 the column event, and was calculated from the dataset of 30 extant teleost species, excluding the

786 missing event data. The individual cells are heatmap color-coded according to percentage value.

787

788 **FIGURE 6 Distribution of heterochronic shifts estimated by event-pairing in the teleost**

789 **phylogeny**

790 (a,b) Scatter plots showing the relationship between the phylogenetic branch length and number

791 of heterochronic shifts estimated from the extant and ancestral developmental sequences by

792 event-pairing with acctran (a) and deltran (b) optimizations (black circle). The reference

793 distribution (open circles) hypothesizes a linear correlation between the branch length and shift

794 number. (c) The coefficient of variation for the number of heterochronic shifts in a branch. The

795 black and open circles show the observed and reference values, respectively. The vertical bars

796 indicate 95% confident intervals for the reference values. The branches are categorized into
 797 three different groups: all branches, branches shorter than 50 million years (Mya), and branches
 798 shorter than 20 Mya. (d) The number of branches without heterochronic shifts calculated from
 799 observed (black circle) and reference (open circle) data in three different branch length
 800 categories, as explained in (c). Vertical bars indicate 95% confident intervals of the reference
 801 value. The number of branches in each analysis were 59 (all branches), 33 (shorter than 50
 802 Mya), and 19 (shorter than 20 Mya).

803

804

805 **FIGURE 7 Comparison of the distribution of heterochronic rank changes estimated by**
 806 **different methods**

807 (a to c) Scatter plots showing the relationship between branch length and the amount of total
 808 rank changes estimated to have occurred in the branch. The ancestral developmental sequences
 809 were estimated by event-pairing with acctran (a) and deltran (b) optimization, and continuous
 810 analysis (c). (d) The number of total rank changes divided by the number of branches. The
 811 coefficients of variation: 0.6405 (continuous), 0.6130 (acctran), and 0.8116 (deltran). (e) The
 812 number of total rank changes per branch per million years. The coefficients of variation: 1.7396

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

Taxon	Common name	Reference	Ranks of events																
			es	op	fs	tb	kv	tbr	ot	le	tl	hb	olf	mff	oto	bc	pfb	ep	h
<i>Amia calva</i>	Bowfins	Ballard, 1986	1	2	2	2	?	3	4	5	5	5	5	6	8	7	7	7	10
<i>Alosa sapidissima</i>	American shad	Shardo, 1995	1	4	2	5	?	3	5	5	6	7	5	6	7	7	7	8	10
<i>Catostomus commersoni</i>	White sucker	Long and Ballard, 1976	1	3	2	3	4	3	4	5	5	6	7	6	7	7	8	8	10
<i>Danio rerio</i>	Zebrafish	Kimmel et al., 1995	1	4	3	2	4	5	5	6	5	8	7	7	7	9	9	8	10
<i>Cyprinus carpio</i>	Common carp	Verma, 1970	1	3	4	?	5	2	5	6	6	7	7	11	8	8	9	8	10
<i>Carassius auratus</i>	Minnnows	Tsai et al., 2013	1	3	3	2	4	4	5	5	5	6	7	5	?	?	6	6	8
<i>Barbodes gonoprokto</i>	Stinging catfish	Puvion-Charvillat et al., 2009	1	2	2	2	4	3	6	5	4	5	5	6	?	?	9	8	10
<i>Heteropneustes fossilis</i>	African catfish	Chen et al., 2010	1	2	2	2	4	3	6	5	4	5	5	6	?	?	9	8	10
<i>Oncorhynchus mykiss</i>	Rainbow trout	Ballard, 1973	1	3	2	?	2	3	7	5	6	6	4	7	9	7	7	8	12
<i>Salmo salar</i>	Atlantic salmon	Pelluet, 1944, Gorodilov, 1996	1	3	2	6	2	?	4	5	?	7	8	8	9	8	9	10	12
<i>Galaxias maculatus</i>	Common galaxias	Benzie, 1968	?	1	2	1	2	1	3	2	5	2	3	7	8	?	6	4	10
<i>Gadus morhua</i>	Atlantic cod	Hall et al., 2004	1	2	1	3	3	3	4	6	?	7	5	4	5	8	8	9	10
<i>Gobius niger</i>	Black goby	Ballard, 1969	1	2	2	3	3	6	5	4	4	8	?	5	7	9	8	8	10
<i>Leucopsarion petersii</i>	Ice goby	Arakawa et al., 1999	1	3	3	2	3	4	5	6	5	7	?	?	9	8	10	8	12
<i>Gasterosteus aculeatus</i>	Three-spined stickleback	Swarup, 1958	1	3	4	?	5	2	5	5	?	6	6	?	6	8	9	7	11
<i>Stizostedion vitreum</i>	Walleye	McElman and Balon, 1979	1	2	3	2	3	4	4	5	6	7	4	6	8	9	11	10	13
<i>Channa striatus</i>	Striped snakehead	Marimuthu and Haniffa, 2007	1	2	2	2	4	3	3	4	4	5	5	4	6	5	7	?	6
<i>Anabas testudineus</i>	Climbing gouramies	Zalina et al., 2012	1	3	4	?	2	4	4	5	?	6	?	?	7	7	?	10	8
<i>Amphilophus xiloensis</i>	Cichlids	Kratochwil et al., 2015	1	2	2	2	?	5	2	4	3	5	?	?	?	7	8	8	6
<i>Cichlasoma dimerus</i>	South American cichlids	Mejide and Guerrero, 2000	1	4	3	2	?	5	5	5	6	5	?	8	6	6	9	8	7
<i>Oreochromis niloticus</i>	Nile tilapia	Fujimura and Okada, 2007	1	3	2	3	?	4	5	6	5	6	6	7	8	8	8	9	10
<i>Labetropheus trewasae</i>	Scrapemouth mbuna	Balon, 1977	1	2	4	3	?	2	3	5	5	6	11	9	5	7	9	8	10
<i>Haplochromis piceatus</i>	Victoria cichlids	de Jong et al., 2009	1	2	2	4	?	4	3	4	5	5	4	6	6	6	7	7	8
<i>Melanotaenia splendida</i>	Eastern rainbow fish	Humphrey et al., 2003	1	2	4	3	3	?	6	5	6	7	?	10	8	7	9	5	11
<i>Adinia xenica</i>	Diamond killifish	Cunningham and Balon, 1985	1	2	3	?	3	2	3	5	?	6	8	9	4	7	8	9	12
<i>Fundulus heteroclitus</i>	Mummichog	Armstrong and Swope Child, 1965	1	2	4	?	3	3	5	5	7	6	5	?	8	7	9	10	12
<i>Xiphophorus maculatus</i>	Southern platyfish	Tavolga and Rugh	1	2	2	4	?	3	3	5	4	5	6	?	8	6	5	7	11
<i>Austrofundulus myersi</i>	Rivulines	Wourms, 1998	1	4	3	2	2	5	6	7	10	7	?	11	9	8	9	10	14
<i>Oryzias latipes</i>	Japanese ricefish	Iwamatsu, 2004	1	3	4	?	2	5	4	6	9	7	13	12	8	8	10	11	16
<i>Oryzias javanicus</i>	Javanese ricefish	Iwamatsu and Hirata, 1984	1	3	4	?	2	5	5	6	9	7	7	?	8	8	9	10	13

Figure 1.

Figure 2.

Figure 3.

Figure 4

Figure 5.

Figure 6.

Figure 7.