Comparative Gene Analysis in Chordate Embryonic Development Reveals Co-existence of Von Baer's and Haeckel's Postulates

Song Guo

Skolkovo Institute of Science and Technology

Haiyang Hu

CAS Key Laboratory of Computational Biology, CAS-MPG Partner Institute for Computational Biology

Chuan Xu

CAS Key Laboratory of Computational Biology, CAS-MPG Partner Institute for Computational Biology

Naoki Irie

University of Tokyo <https://orcid.org/0000-0002-6720-381X>

Philipp Khaitovich (✉ khaitovich@eva.mpg.de)

Skolkovo Institute of Science and Technology

Article

Keywords: evolution, evo-devo, Haeckel, von Baer, development

Posted Date: June 17th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-579345/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

**Comparative gene analysis in chordate embryonic development
reveals co-existence of Von Baer's and Haeckel's postulates**

Song Guo^{1,2,†}, Haiyang Hu^{2,†}, Chuan Xu^{2,†}, Naoki Irie^{3,4}, Philipp Khaitovich^{1,*}

¹ Skolkovo Institute of Science and Technology, 121205, Moscow, Russia

² CAS Key Laboratory of Computational Biology, CAS-MPG Partner Institute for
Computational Biology, Shanghai Institute for Nutrition and Health, CAS, 320 Yue
Yang Road, 200031, Shanghai, China.

³ Department of Biological Sciences, University of Tokyo, Tokyo, Japan.

⁴ Universal Biology Institute, The University of Tokyo, Tokyo, Japan.

[†] Equally contributed to this work.

* Correspondence should be addressed to: P.Khaitovich@skoltech.ru

Abstract

The relationship between embryonic development and evolution historically investigated based on embryo morphology could now be reassessed using mRNA expression endophenotype. Here, we analyzed the conservation of divergence of the developmental mRNA expression profiles in nine evolutionarily distinct species, from oyster to mouse, and compared them to the original concepts formulated by von Baer and Haeckel. We find nearly linear conservation of species' developmental programs among these species, compatible with models rooted on von Baer's postulates, for approximately a third of expressed orthologous genes. By contrast, 5-15% of developmental expression profiles, enriched in neural genes, displayed an alignment pattern compatible with the terminal edition paradigm proposed by Haeckel. Thus, the development-evolution relationship based on mRNA expression agrees with early concepts based on embryo morphology and demonstrates that the corresponding patterns coexist in chordate development.

Keywords: evolution, evo-devo, Haeckel, von Baer, development

Introduction

The general relationship between ontogeny and phylogeny, or development and evolution, has long been discussed. Among cornerstones of the evolutionary developmental theory are the arguments formulated in von Baer's laws of embryology and Ernst Haeckel's Biogenetic law. Although von Baer did not accept the concept of evolution, his idea that the earlier stages of embryogenesis reflect shared traits of a broader taxonomic group¹ laid the foundation for the current evolutionary views on developmental programs' conservation. Ernst Haeckel, who advocated evolution, proposed in his Biogenetic law that the embryogenesis replays the species' evolutionary past. Thus, according to this concept, the new developmental stages will be added to the ancestral embryonic program to produce more recently evolved species². After a long debate, the general concept of early embryogenesis conservation, reflecting a more ancestral stage, continued in a form of a monotonic developmental conservation model known as the funnel model. More recently, a developmental conservation model called the developmental hourglass model was proposed, which placed the most significant stage conservation at a mid-embryonic part of embryogenesis, defined as the phylotypic period³. The applicability of the hourglass model to the conservation of developmental stages at mRNA expression level has been supported both in animals^{4, 5, 6, 7, 8} and plants^{9, 10, 11}. Recent studies, however, demonstrated the applicability of the funnel model or the coexistence and validity of both funnel and hourglass models at different levels of trait evolution in

vertebrates^{8, 12, 13, 14, 15, 16, 17, 18}. Even the broadly criticized Biogenetic law, has been pointed out to have potential applicability of its principles¹⁹.

One of the major barriers in testing the relationship between development and evolution, including the applicability of concepts proposed in Biogenetic law or the von Baer's law of embryology, is the difficulty in identifying evolutionarily-homologous developmental stages among distant species. While pioneering studies focused on the investigation of embryonic morphology²⁰, recent works relied on developmental changes in mRNA expression profiles as an informative endophenotype^{16, 21, 22, 23}. The use of gene expression facilitates inter-species comparisons, as orthologous genes can be matched among species, and their expression profiles could be traced at all stages of development. Several studies examined developmental gene expression patterns in mammals²⁴, vertebrates¹⁶, chordates^{17, 22}, and fruit fly species²⁵. These studies, however, either focused on gene expression patterns within a specific species or compared developmental stage conservation within the concepts of the hourglass model paradigm.

Here, we compared developmental expression patterns among eight chordate species and oyster to directly investigate the compatibility of developmental expression patterns with predictions of the Biogenetic law and von Baer's law of embryology. To enable such a comparison, we formulated a "continuous" developmental model describing the ontogenetic gene expression pattern alignment, which presumes the existence of a general developmental program spanning the entire

development, as suggested by the Baer's law and its later modifications. By contrast, an alternative "progressive" developmental model describes the developmental gene expression profiles of a species with more ancestral body plan organization align the best to early embryonic stages of species with more complex structural organization, as mentioned in Haeckel's Biogenetic law.

Results

Alignment of developmental gene expression patterns among species

We investigated the relationship between ontogeny and phylogeny of chordate species at the level of molecular phenotype using RNA-sequencing (RNA-seq) data collected over the entire course of embryonic development in eight chordate species of different organizational complexity (amphioxus, ciona, zebrafish, two species of frogs, turtle, chicken, and mouse) and an outgroup species (oyster)^{8, 26}. In each species, the data were collected at 11 to 20 developmental stages and measured in duplicates (Fig. 1a; Supplementary Data 1 and Supplementary Fig. 1).

Within species, differences among developmental stages explained approximately 90% of total expression variation (Fig. 1b,c and Supplementary Fig. 2). By contrast, other factors, such as biological replicates and sequence coverage, explained 1% and 0.6% of variation each (Fig. 1b). Accordingly, on average, 85% of detected genes showed significant expression differences among developmental stages in each

species (development-related genes) (polynomial test, Benjamini-Hochberg (BH) corrected $p < 0.05$; Fig. 1d and Supplementary Data 2).

We searched for the best temporal alignment between each pair of species using genes preferentially expressed at a particular developmental stage (stage-associated genes; Methods; Supplementary Data 3)²¹. Surprisingly, despite substantial differences in organizational complexity, an approximately linear alignment of developmental stages was a dominant stage-matching pattern in each pair of species (Fig. 1e and Supplementary Fig. 3,4).

Visualization of developmental expression patterns

We next investigated developmental expression trajectories of the 244 orthologous genes classified as development-related in all nine species using the nearly linear, species' alignment (Fig. 2a). These genes were grouped into six clusters based on their developmental profiles in the unsupervised clustering analysis (Fig. 2b). Remarkably, 66% of the genes fell within a single cluster representing a decreasing expression pattern conserved across all nine species (CL9.1) (Fig. 2c). By contrast, the expression trajectories in the other clusters differed more among species, with the extent of differences being directly proportional to corresponding phylogenetic distances (Fig. 2e and Supplementary Fig. 5).

The same analysis conducted using 2,038 development-related orthologs shared among six vertebrate species revealed a CL9.1-like developmental pattern represented by a single cluster (CL6.1) (Fig. 2d). Genes in clusters CL9.1 and CL6.1 overlapped

significantly and were enriched in similar biological processes, including spliceosome and RNA processing (Fig. 2f and Supplementary Data 4).

The second-largest cluster found in vertebrates (CL6.2) showed an opposite, ascending expression pattern conserved among species and contained genes enriched in signaling pathways, such as cGMP-PKG signaling pathway, oxytocin signaling pathway, and Renin secretion (Supplementary Data 4). Notably, this cluster overlapped with the chordate cluster CL9.4, where the ascending pattern was conserved among the seven species, excluding ciona and oyster (Fig. 2c). By contrast, genes in other clusters showed developmental profiles divergent among species, including patterns involving partially similarity, development shifts, and inversions.

Two alternative evolutionary models of species' development

We next sought to test, from the perspective of gene expression, two models, the continuous and progressive, describing embryogenesis rooted in von Baer's and Haeckel's ideas. Specifically, developmental concepts derived from von Baer's ideas, such as the hourglass and the funnel models, presume general preservation of the developmental profiles across species (Fig. 3a). Our continuous model (CM) follows this assumption, predicting the best alignment between complete developmental expression profiles of the two species. Alternatively, based on the Haeckel's postulate that embryogenesis replays the evolutionary history of the species, we proposed a progressive model (PM), on which the developmental gene expression profiles of a species with more ancestral body plan organization will align the best to early

embryonic stages of species with more recently evolved organization, "replaying" the evolutionary history of this body plan (Fig. 3a; Supplementary Fig. 6).

We found that out of all development-related genes that aligned significantly to the mouse developmental trajectory, 19-36% aligned linearly, thus behaving in agreement with the continuous model predictions (CM genes) (correlation test, $r > 0.7$, $p < 0.05$; Fig. 3b,e; Supplementary Fig. 7 and Supplementary Data 5). By contrast, 5-15% of development-related genes aligned significantly better to the truncated sets (PM genes) (permutations, BH-corrected $p < 0.05$; Fig. 3b,c,e; Supplementary Fig. 7 and Supplementary Data 6). Further, in each pairwise alignment, PM genes did not align uniformly to all shortened mouse developmental intervals but tended to peak at a particular fragment. For instance, oyster PM genes aligned best to the mouse developmental fragments ending at stages 3-4, while amphioxus PM genes – to the fragments ending at stages 5-6 (Fig. 3b,c and Supplementary Fig. 8). Overall, PM genes in species evolutionarily proximal to the mouse tended to align to increasingly longer truncated mouse developmental sets (Fig. 3d). This phylogenetic ordering of ontogenetic patterns was not caused by the alignment procedure, which was not biased to any developmental stage, and robust to the use of evolutionarily old genes present in each of the nine investigated species (Supplementary Fig. 9). By contrast, repeating the alignment procedure using species with more ancient body plans, such as amphioxus or ciona, instead of the mouse, did not yield a consistent significant excess of genes following PM predictions (Supplementary Fig. 10). This phenomenon

suggested that genes conforming to PM predictions demonstrate the phylogeny-ontogeny relationship aligning with the original Haeckel's ideas, even though we did not include this aspect of the Biogenetic law in PM model formulation.

Properties of CM and PM genes

While we defined CM and PM gene sets independently for each species, each set overlapped significantly between species (Methods, subsampling, Bonferroni-corrected $p < 0.05$; Supplementary Data 7), indicating the conservation of CM and PM patterning across chordate embryogenesis and presumably convergent functionality specific to each model.

Indeed, CM genes showed enrichment in Gene Ontology (GO) terms corresponding to general cellular functions, such as spliceosome, RNA transport, DNA replication, and several metabolic processes (Hypergeometric test, $p < 0.05$; Supplementary Table 8). By contrast, PM genes were primarily enriched in neural functions, including axon guidance, glutamatergic synapse, dopaminergic synapse, and MAPK signaling pathways (Hypergeometric test, $p < 0.05$; Fig. 3f and Supplementary Data 8). In line with functional enrichment results, expression of CM genes displayed significantly lower tissue-specificity in mouse²⁷ compared to PM genes, and all expressed genes (one-sided Fisher's exact test, $p < 0.05$; Supplementary Fig. 11). Nonetheless, PM genes were more conserved at the amino acid sequence level compared to CM genes (Ka/Ks, one-sided Wilcoxon rank-sum test, $p < 0.0005$) (Fig. 3g and Supplementary Fig. 12,13). Accordingly, CM genes substantially overlapped with CL9.1 genes

(one-sided Fisher's exact test, odds ratio = 3, $p < 0.0001$) and were involved in the same biological processes as CM genes, including spliceosome and RNA processing (Supplementary Data 9).

Discussion

Our goal was to explore the general relationship between ontogeny and phylogeny based on the comparison of developmental gene expression trajectories among nine species separated by approximately 800 million years of evolution. Our results indicate that the continuous and progressive developmental patterns rooted in von Baer's and Haeckel's ideas match approximately half of all detected mRNA developmental expression profiles. Between the two models, the continuous developmental model consistent with Baer's arguments was nearly three-fold more prominent compared to the progressive model compatible with the Biogenetic law. These results do not conflict with the hourglass model of development. Our previous work demonstrated the applicability of this model to all nine species' development⁸. While the hourglass model compares relative conservation of developmental stages, we focused on the alignment of expression differences among stages.

Expression profiles conserved along the entire species' development included two main patterns: a gradual decrease conserved among all nine species and gradual increase conserved among six vertebrates. The conserved downward pattern coincides with the reported preferential expression of evolutionary older genes at earlier developmental stages²⁸. Consistently, genes forming this pattern tend to display

ubiquitous expression among tissues and are involved in essential functions, such as RNA processing. Further, a study examining embryonic gene expression reported the existence of such a pattern in isolated mouse tissues²⁹, indicating that the descending expression reflects alterations within embryo tissues rather than changes in embryo composition. Overall, expression patterns conserved between species over the entire development involve 19-36% of orthologous development-related genes detected in our study, constituting the major alignment trend.

The second prominent type of developmental relationship among species, involving 5-15% of orthologous development-related genes, reflected the predictions of Haeckel's Biogenetic law. Consistent to Haeckel's views, the best developmental alignment matched the stages reflecting the phylogenetic history of the species, rather than the entire developmental span. When aligning developmental profiles of eight species to the mouse, we indeed observed this phenomenon. First, for this gene group, the developmental expression profiles indeed aligned the best to the truncated mouse developmental series lacking late stages. Second, for each species, these alignments peaked at a particular shortened mouse developmental fragment, with species phylogenetically more distant from the mouse aligning best to the increasingly shorter mouse developmental sets. Intriguingly, while all species evolved their developmental programs after branching from the common ancestor, a mirror procedure, alignment to the fragmented developmental series of amphioxus and ciona representing more ancient body plans, revealed potential PM genes in only two of 16 alignments

(Supplementary Fig. 10). Further, there was no relationship between the length of the best-aligning developmental fragment and the phylogenetic distance between species.

Similar to the linearly-aligning genes, the expression behavior of progressive genes overlapped significantly between species (Supplementary Data 7), indicating their potential functional conservation. These genes were indeed more conserved at the amino acid sequence level than the linearly-aligning genes and displayed enrichment in specialized functions, such as synaptic signaling and secretion.

Our work highlights the usefulness of quantitative endophenotypes as an essential complement to morphology-based developmental studies. It further suggests partial applicability of Haeckel's ideas to a fraction of developmental expression differences involving genes associated with neural functions.

Methods

RNA-seq data processing

All data in this analysis were downloaded from Hu et al.⁸. We adopted the same methods to quantify gene expression. In brief, we got the RNA-seq data of *Crassostrea gigas* from GEO database (accession number SRP014559)²⁶, and the rest of species from DDBJ (accession number DRA003460) (Supplementary Data 1). To quantify gene expression, we mapped RNA-seq reads to the corresponding genome (Supplementary Data 1) using Tophat (v2), allowing up to three mismatches and indels, except for ciona (Ci). In the case of ciona, we mapped reads to the genome

with up to five mismatches and indels as the ciona RNA-seq data and the genome data have slightly lower quality compared to the rest.

To define the expressed genes, we required the maximal expression across all development stages exceeding 1 FPKM. For each stage, we calculated expression as the mean of expression of the replicated samples. More than 70% of coding genes annotated for a given species were reliably detected, except for amphioxus (41%) (Supplementary Data 10).

Identification of development-related genes

We defined developmental alterations of gene expression levels using polynomial regression models following the method described in Somel et al.³⁰. For each gene, we chose the best regression model with the developmental stage (by rank) as predictor and expression level as a response with Benjamini-Hochberg corrected $p < 0.05$. The genes that fit a significant regression model were termed as development-related genes (Supplementary Data 2).

Identification of stage-associated genes

We defined genes preferentially expressed at a particular developmental stage as stage-associated genes in each species following the method described in^{8, 21}. For each species, we required FPKM (fragments per kilobase of exon model per million reads mapped) > 2 at a particular stage and Z-score (normalized FPKM across samples) representing the difference between this stage and the rest of stages > 1.5 . On average,

65%-90% of genes expressed in development were identified as stage-associated genes (Supplementary Data 3).

Alignment of mouse developmental stage sets to developmental profiles of other species

To assess the transcriptome similarity in the course of developmental stages between mouse (Mm) and other species, we used stage-associated and 1:1 orthologous gene to align the developmental stages between mouse and other species followed by the method reported in^{8, 21}. The detailed description can be found in the supplementary materials of^{8, 21}. In brief, we calculated the pairing score using the hypergeometric test to evaluate the ratio of orthologs overlapping within the stage-associated genes pairwise. The pairing score can be written as:

$$\text{Paring score} = -\log_{10}(\text{Bonferroni corrected P-values})$$

The paring score was used to quantify the significance of the overlap on each pairwise stage comparison between the mouse and another species. From the paring score, we identified the relationship between mouse and other species (Fig. 1e). To check the stability of this relationship, we repeated the comparison 500 times with randomly assigned stage-associated genes for each stage of each species using the same procedure.

284

285 According to the above scheme, we further assigned the corresponding stage
 286 alignment between mouse and other species using the Needleman-Wunsch algorithm
 287 with the gap penalty equal to one. To estimate the stability of alignment based on the
 288 mean of the replicates, we randomly chose one individual per stage, aligned two
 289 species 500 times, and calculated the frequency for each pair of alignment. The
 290 resulting frequency was presented by the thickness of the line in Fig. 2a.

291 *Identification of CM/PM genes*

292 To identify genes with expression trajectories compatible with the continuous model
 293 (CM) or progressive model (PM) predictions, we considered development-related
 294 genes as mentioned in “*Identification of development-related genes*” with existing
 295 orthologs between mouse and any other species. We then linearly aligned the
 296 complete set of developmental stages in a given species (oyster, amphioxus, ciona,
 297 zebrafish, frogs, turtle, and chicken) to the increasingly truncated sets of mouse
 298 developmental stages, i.e., from first two to all 17 stages (Supplementary Fig. 6). To
 299 this end, we interpolated 20 time points uniformly distributed across the whole
 300 ontogeny of non-mouse species using cubic smoothing spline (with three degrees of

freedom for amphioxus data and six degrees of freedom for other species) or polynomial regression curves (up to the fourth degree). The two methods for interpolating expression values across development were used to ensure the robustness of our CM or PM predictions. Of note, during cubic smoothing spline, the degrees of freedom chosen for amphioxus were fewer than other species, due to the relatively fewer sampled developmental stages of this species; whereas during polynomial regression, we chose up to four degrees to take advantage of the near-fitness of polynomial regression in most time series data.

We then aligned the resulting expression trajectories to the gradually increasing sets of mouse developmental stages, where the expression trajectory was interpolated using the same approach (cubic smoothing spline with six degrees of freedom or polynomial regression with up to the fourth degree). The alignments with the Pearson correlation coefficient (PCC) greater than 0.7 and correlation test p -value less than 0.05 were considered as valid. We further selected the alignment with the maximum PCC among all valid alignments as the ultimate one. Genes failing to pass the two alignment criteria were considered as non-aligned, regardless of the complete or partial linear alignment paradigms.

Based on the above classification procedure, we summarized for each species, the number of genes aligning to each set of developmental stages of the mouse. Furthermore, we repeated the classification process by shuffling the orthologous relationships between non-mouse species and the mouse (Supplementary Fig. 7).

Specifically, we expected that by shuffling, each developmental stage of the mouse would be equally represented in other species, allowing us to calculate the random expectation of alignment occurrences for each stage. Thus, the final number of genes aligning to each set of developmental stages of the mouse was obtained after subtracting these background contaminations. Genes adhering to the continuous model (CM) were defined as those best aligned between mouse and other species over the complete developmental intervals. By contrast, genes following the progressive model (PM) were defined by the following criteria: i) aligned best to a truncated set of mouse developmental stages; ii) displayed a significantly greater number of aligning genes compared to the background distributions, determined using a BH-corrected p -value of less than 0.05 as a cutoff; and iii) an increase in the number of genes aligning to particular sets of mouse stages compared to its adjacent sets (Fig. 3a, Supplementary Data 5 and Supplementary Data 6).

To further check the robustness of the alignment results, we restricted our analysis to evolutionarily old genes. To do so, we repeated the alignment procedure using a subset of the development-related genes with inferred Earliest Ortholog Level (EOL) < level 10. This conservation level corresponds to genes that appeared and became fixed within the genomes before the separation of Chordata³¹.

Overlap of CM/PM genes between species

To calculate the significance of the overlap of CM or PM genes between species, we sampled the same number of CM or PM genes in each species from all

development-related genes 1,000 times and recalculated the number of overlapping genes to obtain the empirical distribution. The overlap significance p -value was calculated as the proportion of the values, which were as larger or greater than the actual overlapping gene number. Given all pairs of species involved, a Bonferroni-corrected p -value of less than 0.05 was used as the cutoff of the overlap significance (Supplementary Data 7).

Amino acid conservation of CM/PM gene

We compared the evolutionary conservation between proteins encoded by CM and PM genes using the Ka/Ks metric³². Since in this study, we are not studying Ka/Ks variations across the evolutionary branches – we focused on the comparisons of PM versus CM genes within each individual species, we conducted this analysis mainly in a pairwise manner between a given species and mouse. Firstly, we extracted the coding sequences of each gene based on the corresponding annotation information and then translated the sequences into amino acid sequences using the function "translate" implemented in the Bioconductor package "Biotstrings". The longest protein sequence was selected as the gene protein sequence. Next, we performed protein sequence alignments between non-mouse species and the mouse using the function "pairwiseAlignment" in "Biostrings", with the scoring matrix set as BLOSUM50. The resulting protein alignment was translated to the nucleotide level and used as the input for PAML³³ for a Ka/Ks analysis output. This procedure was conducted in a pairwise manner for the mouse and a given non-mouse species to match the corresponding

definitions of PM and CM genes carried out in a pairwise manner (Supplementary Fig. 12). To test the robustness of our Ka/Ks calculations in the context of multiple species, we performed multiple sequence alignment using Clustal Omega³⁴ for all protein sequences mentioned above convert to nucleotide levels. We next utilized the CODEML application in PAML to calculate the Ka/Ks for given cross-species ortholog. The significance of the difference between PM and CM genes in each species was assessed using one-sided Wilcoxon rank-sum test (Supplementary Fig. 13).

Gene expression interpretation to mouse developmental stage

To compare the similarity of the expression profiles across developmental stages of nine species, we used the predicted developmental stage alignment presented in Fig. 2a to create a unified alignment of eight species to the mouse developmental curves. To do so, we mapped 33 stages cumulatively interpolated from eight species to the full mouse developmental curve fitted using cubic smoothing spline with ten degrees of freedom. We then compared gene expression curves among nine species based on z-transformed expression of each gene interpolated at these 33 stage points.

Clustering of gene expression profiles in six vertebrate and nine chordate species

To investigate the expression pattern diversity in nine or six species, we used hierarchical clustering (hclust function in R) of z-transformed gene expression trajectories aligned among species with (1 - rho) as the distance measure, where rho is

the Spearman correlation coefficient. We chose k equal six, as optimal, based on visual inspection of clusters obtained using different k values.

Functional annotation of developmental expression patterns

To check the functions of genes in each cluster, we applied Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)³⁵ enrichment tests. GO annotation of mouse genes was downloaded from Bioconductor package "org.Mm.eg.db". Mouse pathway annotation was downloaded from <http://rest.kegg.jp/list/pathway/> and <http://rest.kegg.jp/link/genes/>.

For GO enrichment test, the "elim" algorithm of topGO³⁶ was chosen to eliminate the hierarchical dependency of the GO terms. Fisher's exact test was applied for each GO term. The background set consisted of all development-related genes orthologous among six vertebrate species (2,038 genes) and nine chordate species (244 genes), respectively. For the test of vertebrate gene clusters, Benjamini-Hochberg correction on "molecular function", "biological process", and "cellular component" was applied independently. GO terms with BH corrected $p < 0.05$ were reported. We applied no multiple test correction to chordate' gene cluster enrichment analysis due to low statistical power of the dataset and used a more stringent nominal p -value cutoff of $p < 0.001$.

For the pathway enrichment test, the reference genes were the same as for GO-based analysis. We used hypergeometric test (phyper in R) to assess the enrichment in each KEGG pathway. Bonferroni correction was applied for genes in

vertebrate clusters. Pathways with corrected $p < 0.05$ were reported as significantly enriched. No correction was applied to genes from chordate clusters due to low statistical power, and the nominal pathway enrichment p -value was set to $p < 0.05$. This relaxed cutoff was used to assess the potential overlap between enriched functions found in vertebrate and chordate clusters (Supplementary Data 4).

Species tree construction

The species tree was generated under the web <https://phylot.biobyte.de/> with NCBI taxonomy IDs (10090, 9031, 13735, 8364, 7955, 7719, 8355, 7739, 29159).

The species separation time to the mouse was obtained from <http://www.timetree.org/>³⁷.

Statistical analysis and software

Analyses were conducted in the R environment (<http://www.r-project.org/>). To minimize the type I error rate, we used multiple test correction for p -value calculations, unless specifically indicated otherwise. The statistically significant level used for each was specified in the main text. Additionally, we used Perl, Python, and R packages, including "topGO", "reshape", "RColorBrewer", "ggplot2", "Biotrings", as well as shell scripts, in the analyses.

Data availability statement

The RNA-seq data of *Crassostrea gigas* were from GEO database (accession number SRP014559)²⁶, and the rest of species from DDBJ (accession number DRA003460)

(Supplementary Data 1). All the codes used have been uploaded to GitHub:

<https://github.com/guosongcn/evo-devo>

Acknowledgments

We thank Michael Lachmann for inspiring this study, Zhisong He, Qian Li and

Qianhui Yu for helpful discussions, and Pavel Mazin for critical comments.

Funding

This work was supported by the National Natural Science Foundation of China

(Grants 31420103920 and 91331203 to P.K.); the National One Thousand Foreign

Experts Plan (Grant WQ20123100078 to P.K.); and Grant-in-Aid for Scientific

Research on Innovative Areas (17H06387 to N.I.).

Author contribution

S.G, HY.H and C.X designed and executed the bioinformatics analysis. S.G and PK

wrote the manuscript. N.I and P.K revised the manuscript. PK supervised the project.

Conflict of interest

The authors declare no conflict of interest.

References

1. Von Baer KE. *Über Entwicklungsgeschichte Der Thiere.* (1828).
2. Haeckel E. *Generelle Morphologie der Organismen. Berlin: Georg Reimer,* (1866).
3. Duboule D. Temporal colinearity and the phylotypic progression: a basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony. *Dev Suppl*, 135-142 (1994).
4. Domazet-Loso T, Tautz D. A phylogenetically based transcriptome age index mirrors ontogenetic divergence patterns. *Nature* **468**, 815-818 (2010).
5. Kalinka AT, *et al.* Gene expression divergence recapitulates the developmental hourglass model. *Nature* **468**, 811-814 (2010).
6. Irie N, Sehara-Fujisawa A. The vertebrate phylotypic stage and an early bilaterian-related stage in mouse embryogenesis defined by genomic information. *BMC Biol* **5**, 1 (2007).
7. Irie N, Kuratani S. Comparative transcriptome analysis reveals vertebrate phylotypic period during organogenesis. *Nat Commun* **2**, 248 (2011).
8. Hu H, *et al.* Constrained vertebrate evolution by pleiotropic genes. *Nat Ecol Evol* **1**, 1722-1730 (2017).
9. Quint M, Drost HG, Gabel A, Ullrich KK, Bonn M, Grosse I. A transcriptomic hourglass in plant embryogenesis. *Nature* **490**, 98-101 (2012).
10. Cheng X, Hui JH, Lee YY, Wan Law PT, Kwan HS. A "developmental hourglass" in fungi. *Mol Biol Evol* **32**, 1556-1566 (2015).

11. Cridge AG, Dearden PK, Brownfield LR. Convergent occurrence of the developmental hourglass in plant and animal embryogenesis? *Ann Bot* **117**, 833-843 (2016).
12. Bininda-Emonds OR, Jeffery JE, Richardson MK. Inverting the hourglass: quantitative evidence against the phylotypic stage in vertebrate development. *Proc Biol Sci* **270**, 341-346 (2003).
13. Roux J, Robinson-Rechavi M. Developmental constraints on vertebrate genome evolution. *PLoS Genet* **4**, e1000311 (2008).
14. Artieri CG, Haerty W, Singh RS. Ontogeny and phylogeny: molecular signatures of selection, constraint, and temporal pleiotropy in the development of *Drosophila*. *BMC Biol* **7**, 42 (2009).
15. Comte A, Roux J, Robinson-Rechavi M. Molecular signaling in zebrafish development and the vertebrate phylotypic period. *Evol Dev* **12**, 144-156 (2010).
16. Piasecka B, Lichocki P, Moretti S, Bergmann S, Robinson-Rechavi M. The hourglass and the early conservation models--co-existing patterns of developmental constraints in vertebrates. *PLoS Genet* **9**, e1003476 (2013).
17. Levin M, *et al.* The mid-developmental transition and the evolution of animal body plans. *Nature* **531**, 637-641 (2016).
18. Uesaka M, Kuratani S, Takeda H, Irie N. Recapitulation-like developmental transitions of chromatin accessibility in vertebrates. *Zoological Lett* **5**, 33 (2019).
19. Richardson MK, Keuck G. Haeckel's ABC of evolution and development. *Biol Rev Camb Philos Soc* **77**, 495-528 (2002).
20. Jeffery JE, Bininda-Emonds OR, Coates MI, Richardson MK. Analyzing evolutionary patterns in amniote embryonic development. *Evol Dev* **4**, 292-302 (2002).

21. Li JJ, Huang H, Bickel PJ, Brenner SE. Comparison of *D. melanogaster* and *C. elegans* developmental stages, tissues, and cells by modENCODE RNA-seq data. *Genome Res* **24**, 1086-1101 (2014).
22. Yanai I, Peshkin L, Jorgensen P, Kirschner MW. Mapping gene expression in two *Xenopus* species: evolutionary constraints and developmental flexibility. *Dev Cell* **20**, 483-496 (2011).
23. Bozinovic G, Sit TL, Hinton DE, Oleksiak MF. Gene expression throughout a vertebrate's embryogenesis. *BMC Genomics* **12**, 132 (2011).
24. Wagner RA, Tabibiazar R, Liao A, Quertermous T. Genome-wide expression dynamics during mouse embryonic development reveal similarities to *Drosophila* development. *Dev Biol* **288**, 595-611 (2005).
25. Tomancak P, *et al.* Global analysis of patterns of gene expression during *Drosophila* embryogenesis. *Genome Biol* **8**, R145 (2007).
26. Zhang G, *et al.* The oyster genome reveals stress adaptation and complexity of shell formation. *Nature* **490**, 49-54 (2012).
27. Li B, *et al.* A Comprehensive Mouse Transcriptomic BodyMap across 17 Tissues by RNA-seq. *Sci Rep* **7**, 4200 (2017).
28. Gao L, *et al.* Chromatin Accessibility Landscape in Human Early Embryos and Its Association with Evolution. *Cell* **173**, 248-259 e215 (2018).
29. Sarropoulos I, Marin R, Cardoso-Moreira M, Kaessmann H. Developmental dynamics of lncRNAs across mammalian organs and species. *Nature* **571**, 510-514 (2019).
30. Somel M, *et al.* Transcriptional neoteny in the human brain. *Proc Natl Acad Sci U S A* **106**, 5743-5748 (2009).

- 537 31. Litman T, Stein WD. Obtaining estimates for the ages of all the protein-coding genes and
538 most of the ontology-identified noncoding genes of the human genome, assigned to 19
539 phylostrata. *Semin Oncol* **46**, 3-9 (2019).
540
- 541 32. Yang Z, Nielsen R. Estimating synonymous and nonsynonymous substitution rates under
542 realistic evolutionary models. *Mol Biol Evol* **17**, 32-43 (2000).
543
- 544 33. Yang Z. PAML: a program package for phylogenetic analysis by maximum likelihood.
545 *Comput Appl Biosci* **13**, 555-556 (1997).
546
- 547 34. Sievers F, *et al.* Fast, scalable generation of high-quality protein multiple sequence
548 alignments using Clustal Omega. *Mol Syst Biol* **7**, 539 (2011).
549
- 550 35. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids*
551 *Res* **28**, 27-30 (2000).
552
- 553 36. Alexa A, Rahnenfuhrer J. topGO: enrichment analysis for Gene Ontology. R Packag.
554 version 2.26.0. *R Package version 2260*, (2016).
555
- 556 37. Kumar S, Stecher G, Suleski M, Hedges SB. TimeTree: A Resource for Timelines,
557 Timetrees, and Divergence Times. *Mol Biol Evol* **34**, 1812-1819 (2017).
558
559

Figure 1. Species phylogeny and alignment of embryonic development stages.

a Experimental scheme showing the phylogenetic relationship among species (dark blue dendrogram) and numbers of sampled developmental stages. The abbreviations here and throughout the figures indicate species: Cg – oyster (*Crassostrea gigas*), Ci – ciona (*Ciona intestinalis*), Bf – amphioxus (*Branchiostoma floridae*), Dr – zebrafish (*Danio rerio*), Xt – Western clawed frog (*Xenopus tropicalis*), XI – African clawed frog (*Xenopus laevis*), Ps – turtle (*Pelodiscus sinensis*), Gg – chicken (*Gallus gallus*), Mm – mouse (*Mus musculus*).

b Variance explained by different factors based on ANOVA. Boxes represent the interquartile values of the variance proportion explained by the factors listed on the x-axis among nine species. Dots represent the mean explained variance proportion for each species.

c PCA plot based on the expression of 16,685 genes in mouse development. Dots represent samples, and color represents developmental stages (red – early, blue – late).

d The number of detected and development-related genes in each species.

e Heatmap showing the pairing scores reflecting overlap of stage-associate genes (darker shade of blue representing greater overlap) and the optimal alignment path calculated using the Needleman-Wunsch algorithm (black line)

Figure 2. Developmental expression of genes based on the species' alignment.

a Pairwise alignment of developmental stages between mouse and the other species. Thick lines represent alignments supported by technical replicates. Thicker lines represent more stable alignments calculated by random subsampling of samples 500 times. **b** Hierarchical clustering of concatenated developmental gene expression trajectories of 244 gene orthologs shared among nine species. Colors represent clusters. **c** Developmental gene expression patterns in each of six clusters based on nine-species gene orthologs. Colors represent species. Panel titles show cluster identifiers and number of contained genes. **d** Developmental gene expression patterns in each of six clusters based on six vertebrate species gene orthologs. Panel titles

show cluster identifiers and number of contained genes. **e** Relationship between the similarity of developmental gene expression patterns and phylogenetic distances. The expression similarity was calculated as the mean of Spearman correlation coefficients between mouse and non-mouse expression trajectories. **f** Chord graph indicating the relationship between clusters obtained using nine-species and six-species ortholog genes. Wider chords represent stronger connection between clusters.

Figure 3. Relationship of developmental gene expression patterns among species.

a Schematic description of the progressive and continuous models and their predicted alignment patterns. The progressive model (PM, left) involves an extension of developmental program in more recently evolved species, thus predicting the best alignment to a shortened mouse developmental course. The continuous model (CM, right) predicts the best alignment to the complete developmental course.

b Numbers of genes showing the best expression trajectory's alignment between the complete developmental course of the oyster (left), amphioxus (middle), and zebrafish (right) and mouse developmental sets of different lengths: from two to 17 stages. Colors indicate two methods used for developmental expression trajectory calculation: cubic smooth spline (orange) and polynomial regression (gray). Colored rectangles mark stages containing alignments fitting CM (green) or PM (orange) predictions. Black triangles indicate the significantly greater (up) or lesser (down) number of genes than that expected by chance aligning to an indicated mouse developmental interval (permutation test, BH-corrected $p < 0.05$).

c Examples of PM gene expression patterns. Dots show the cluster-level standardized expression levels at each

developmental stage in mouse (black) and the other species (orange). The curves represent average expression profiles, and the shaded regions represent the standard deviation of curve estimates. **d** The relationship between the lengths of truncated sets of mouse stages containing maximal numbers of PM genes for each of the eight species and the phylogenetic distances to the mouse. Dots represent species. The dotted line marks the regression curve. The Pearson correlation coefficient and linear regression p-value are shown at the top right. Mya – millions of years ago. **e** Numbers of CM and PM genes identified in each of the eight species. Note that PM gene number in chicken (Gg) might be inflated due to poor resolution of PM and CM predictions at close phylogenetic distances. **f** KEGG pathways significantly enriched in PM or CM from all eight non-mouse species. The heat map shows the p-values of the enrichment tests. Colored rectangles mark significantly enriched KEGG pathways. **g** Amino acid sequence conservation (Ka/Ks) of PM and CM genes from all eight non-mouse species. Asterisks indicate the significance of the difference (Wilcoxon rank-sum one-sided test, $p < 0.0005$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFigurecb.pdf](#)
- [SupplementaryData1.xlsx](#)
- [SupplementaryData2.xlsx](#)
- [SupplementaryData3.xlsx](#)
- [SupplementaryData4.xlsx](#)
- [SupplementaryData5.xlsx](#)
- [SupplementaryData6.xlsx](#)
- [SupplementaryData7.xlsx](#)
- [SupplementaryData8.xlsx](#)
- [SupplementaryData9.xlsx](#)
- [SupplementaryData10.xlsx](#)