Mammalian skull heterochrony reveals modular evolution and a link between cranial development and brain size

Daisuke Koyabu1,2, Ingmar Werneburg1, Naoki Morimoto3, Christoph P.E. Zollikofer3, Analia M. Forasiepi1,4, Hideki Endo2, Junpei Kimura5, Satoshi D. Ohdachi6, Nguyen Truong Son7 & Marcelo R. Sánchez-Villagra1

The multiple skeletal components of the skull originate asynchronously and their developmental schedule varies across amniotes. Here we present the embryonic ossification sequence of 134 species, covering all major groups of mammals and their close relatives. This comprehensive data set allows reconstruction of the heterochronic and modular evolution of the skull and the condition of the last common ancestor of mammals. We show that the mode of ossification (dermal or endochondral) unites bones into integrated evolutionary modules of heterochronic changes and imposes evolutionary constraints on cranial heterochrony. However, some skull-roof bones, such as the supraoccipital, exhibit evolutionary degrees of freedom in these constraints. Ossification timing of the neurocranium was considerably accelerated during the origin of mammals. Furthermore, association between developmental timing of the supraoccipital and brain size was identified among amniotes. We argue that cranial heterochrony in mammals has occurred in concert with encephalization but within a conserved modular organization.
The mammalian cranium displays great morphological disparity, reflecting the wide spectrum of ecological diversification in the group. A major aspect of skull diversification is heterochrony, or changes in developmental timing, for which genetic mechanisms are now increasingly being understood. Simple alterations in the onset, duration and tempo of development are regarded as causes of profound morphological changes. Until recently, most mammalian heterochronic studies have focused on postnatal life, and our knowledge of fetal development has largely been restricted to model organisms. The critical stages for examination of organogenesis are fetal or around the time of birth, and thus non-model organisms are rarely available and difficult to sample. Recent progress in high-resolution imaging techniques has provided new avenues to non-destructive investigation of fetal and neonatal specimens from museum collections. Microtomographic imaging allows documentation of the onset of individual bone ossification, a powerful marker for tracing perinatal anatomy. This approach has served to identify fundamental differences in postcranial osteogenesis between marsupials and placentals and unsuspected variation in placential development. However, the ancestral patterns of craniogenesis timing and factors behind the cranial heterochrony remain largely unknown. In this study, we show that the timing of bone formation in the mammalian skull is greatly influenced by two factors such as brain size and developmental modularity caused by the mode of ossification.

Encephalization is a central phenomenon in mammalian evolution, one that has led to the largest branched vertebrates, as best exemplified by primates and cetaceans. During the early evolution of mammals in the Jurassic, brain expansion was associated with the acquisition of the neocortex and diversification of sensory faculties. Ontogenetically and evolutionarily, the expansion of the cranial vault reflects brain size increase as also for humans. Given such somatic integration between the skull and the brain, we tested whether the heterochronic changes in embryonic ossification reflect the evolution of brain size. Modularity, referring to the strong internal integration and weak interactions among morphological subsets, is another aspect of patterns of heterochrony to consider. It has been suggested that genetic modularity affects the evolutionary dynamics of species, which in turn influence the evolution of molecular networks regulating morphogenesis. However, the link between heterochrony and modularity in macroevolution remains largely unknown.

A comprehensive sampling of museum collections across the world using non-destructive micro-computed tomography technique produced skeletal developmental sequences for 21 cranial elements of 102 mammalian species and 32 non-mammalian amniote species (sauropterygians). Covering almost all major mammalian groups, our exceptionally large data set was used to reconstruct the developmental sequence of the common ancestor of mammals, and provide insights into evolutionary patterns of skeletal development. Herein, we demonstrate that cranial heterochrony reflects the encephalization history of mammals and conserved modular organization of skull elements.

## Results

### Reconstruction of ancestral ossification sequence

The ancestral conditions and heterochronic changes of ossification sequence at all nodes were reconstructed from developmental sequences of 134 amniote species (Supplementary Data 1 and 2; and Supplementary Table 1) using squared-change parsimony under a Brownian motion model. The ossification sequence inferred for the ancestor of Mammalia is as follows: (1) premaxilla and maxilla, (2) dentary, (3) palatine, (4) frontal and squamosal, (5) pterygoid, (6) jugal, (7) parietal, (8) nasal and ectotympanic, (9) vomer, (10) exoccipital and gonial, (11) basioccipital, (12) lacrimal, supraccoxipital and alisphenoid, (13) basisphenoid, (14) orbitosphenoid and (15) petrosal (Fig. 1 and Supplementary Table 2). Inferred heterochromies for all other nodes are given in Supplementary Figs 1–21. We also conducted an alternative reconstruction by Parsimony-based genetic inference (PGI). This approach treats the sequence as one single, complex character and uses the Parsimov algorithm as an edit-cost function to optimize ancestral states and sequence heterochromies. The inferred sequence for the ancestor of Mammalia is: (1) premaxilla, (2) maxilla, dentary, nasal, jugal, frontal, parietal, squamosal, vomer, palatine and ectotympanic, (3), lacrimal, (4) basioccipital and supraoccipital, (5) pterygoid, (6) basisphenoid, (7) orbitosphenoid, alisphenoid and exoccipital, (8) gonial and (9) petrosal. The PGI sequence was less resolved (that is, involving more tied ranks) than that generated by squared-change parsimony, but both were mostly similar (Spearman’s rank correlation analysis, $r_s = 0.80$, $n = 21$, $P < 0.0001$). Inferred heterochromies for higher taxonomic levels are given in Fig. 2, and those for more inclusive nodes are given in Supplementary Figs 22–29. The results obtained by squared-change parsimony showed that the last common ancestor of Mammalia had a more accelerated onset of ossification of the vomer, frontal, parietal, basioccipital, exoccipital, and supraoccipital compared to non-mammalian amniotes (Supplementary Table 2). The PGI analysis showed that the last common ancestor of Mammalia had a more accelerated onset of ossification of the frontal, parietal, basisphenoid, basioccipital and supraoccipital (Fig. 2).

### Ossification patterns and encephalization

Results of correlation analysis of the relative timing of cranial ossification (scaled from 0 to 1) and encephalization quotient (EQ), which is the residual of
Figure 2 | Heterochronic shifts in the onset of skull bone ossification recovered by the Parsimov-based genetic inference (PGi) analysis in amniotes. Significant shifts detected in derived nodes compared with ancestral nodes are summarized. A, acceleration; D, delay. Numbers in the tree represent the detected cranial elements.
an allometric regression of brain weight against body weight are given in Table 1. We found that the ossification onset of the supraoccipital bone occurs earlier in taxa with higher EQ in mammals (Pearson’s product moment correlation analysis, \( r = -0.65, n = 48, P<0.0001 \)) (Fig. 3). Similarly, among non-mammalian amniotes, a tight correlation was found between the developmental timing of the supraoccipital and EQ (Pearson’s product moment correlation analysis, \( r = -0.95, n = 9, P<0.001 \)). These correlations were similarly significant in the phylogenetically controlled correlation analysis (Fig. 4; Table 1). The results by squared-change parsimony revealed that the developmental timing of the supraoccipital was shown to be significantly earlier in mammals than in non-mammalian amniotes (U-test on phylogenetic independent contrasts, mammals \( n = 79 \), non-mammalian amniotes \( n = 30 \), \( P<0.001 \)).

Modularity analysis. Depending on the anatomical identity of skull bone elements, the cranial region can be divided into different modules. Developmentally, the skull can be divided into mesoderm and neural crest cell-derived elements. Similarly, skull bones are classified into either dermal bones or endochondral bones, depending on their mode of ossification. Morphometric analyses of adult mammalian skulls have previously identified five phenotypic variational modules: oral, zygomatic, nasal, cranial base and cranial vault. We considered these divisions as hypothetical modules, and tested whether these

| Table 1 | Pearson’s correlation coefficients and P-values for comparisons between relative developmental timing and EQ. |
|---|---|---|---|
| Mammals | | | |
| Premaxilla | -0.04 | 0.79 | -0.11 | 0.43 |
| Maxilla | 0.12 | 0.39 | -0.15 | 0.30 |
| Dentary | 0.03 | 0.82 | -0.09 | 0.53 |
| Frontal | -0.03 | 0.85 | -0.11 | -0.41 |
| Nasal | -0.10 | 0.49 | 0.01 | 0.96 |
| Jugal | -0.14 | 0.32 | -0.12 | 0.38 |
| Lacrimal | -0.16 | 0.25 | -0.16 | 0.26 |
| Parietal | 0.10 | 0.44 | 0.22 | 0.11 |
| Squamosal | -0.11 | 0.44 | 0.03 | 0.81 |
| Vomer | -0.02 | 0.87 | -0.10 | 0.48 |
| Palatine | 0.20 | 0.15 | -0.27 | 0.06 |
| Orbitosphenoid | -0.11 | 0.43 | -0.18 | 0.21 |
| Basisphenoid | -0.18 | 0.18 | 0.00 | 1.00 |
| Pterygoid | -0.09 | 0.53 | -0.17 | 0.23 |
| Alisphenoid | -0.07 | 0.61 | 0.00 | 0.98 |
| Basioccipital | -0.01 | 0.94 | -0.03 | 0.83 |
| Supraoccipital | -0.65 | **0.00** | -0.60 | **0.00** |
| Exoccipital | -0.11 | 0.41 | -0.18 | 0.19 |
| Ectotympanic | -0.33 | 0.03 | 0.03 | 0.85 |
| Goniale | -0.49 | **0.00** | -0.17 | 0.28 |
| Petrosal | -0.06 | 0.64 | -0.20 | 0.14 |

| Non-mammalian amniotes | | | |
| Premaxilla | -0.24 | 0.53 | -0.34 | 0.37 |
| Maxilla | -0.29 | 0.45 | -0.49 | 0.18 |
| Dentary | 0.03 | 0.93 | 0.09 | 0.82 |
| Frontal | 0.62 | 0.08 | 0.42 | 0.26 |
| Nasal | -0.50 | 0.21 | -0.26 | 0.54 |
| Jugal | -0.34 | 0.37 | -0.18 | 0.64 |
| Lacrimal | -0.66 | 0.07 | -0.65 | 0.08 |
| Parietal | 0.23 | 0.56 | -0.14 | 0.72 |
| Squamosal | -0.51 | 0.16 | -0.54 | 0.13 |
| Vomer | -0.36 | 0.38 | -0.14 | 0.97 |
| Palatine | -0.68 | 0.04 | -0.73 | 0.03 |
| Orbitosphenoid | | | | |
| Basisphenoid | 0.30 | 0.44 | 0.23 | 0.55 |
| Pterygoid | 0.09 | 0.82 | 0.05 | 0.91 |
| Alisphenoid | | | | |
| Basioccipital | 0.28 | 0.46 | 0.35 | 0.36 |
| Supraoccipital | -0.95 | **0.00** | -0.95 | **0.00** |
| Exoccipital | 0.44 | 0.23 | 0.25 | 0.52 |
| Ectotympanic | -0.48 | 0.23 | -0.44 | 0.28 |
| Goniale | 0.71 | 0.04 | 0.36 | 0.38 |
| Petrosal | 0.43 | 0.25 | 0.36 | 0.35 |

Both raw comparisons and phylogenetically corrected comparisons (Felsenstein’s independent contrasts) are given. Significance level was set as \( P<0.05/21 \) after Bonferroni correction, and statistically significant values are given in bold. Ectotympanic, goniale and alisphenoid of mammals were homologized to angular, prearticular and epitypogryid of non-mammalian amniotes, respectively. Values for orbitosphenoid of non-mammalian amniotes are not available because of uncertain homologies of this bone. Values for alisphenoid (epitypepogyid) of non-mammalian amniotes are not available, as both the ossification timing of this bone and EQ is reported only for one species (Lacerta agilis).
modules are identifiable in the patterns of skeletal heterochrony. Our results on pooled species demonstrated that timing of ossification of dermal bones is skewed towards earlier developmental stages than that of endochondral bones (U-test, n = 1,470, P < 0.05) (Fig. 6a). In addition, neighbour-joining cluster analysis showed that the skull bones form two evident clusters, one cluster consisting explicitly of dermal bones and the other of endochondral bones (n = 1,470) (Fig. 6b,c).

**Discussion**

The common ancestor of Mammalia was found to have an accelerated onset of ossification of the cranial bones associated with the braincase (frontal, parietal, basioccipital and supraoccipital) when compared with non-mammalian amniotes. Morganucodon (Fig. 1), one of the basal-most mammaliaforms (the clade that includes mammals and their closest relatives), had a greatly expanded olfactory bulb, olfactory cortex, neocortex and...
The expanded brain regions are covered by accelerated bones of the skull-roof, suggesting that increased encephalization led to quantifiable developmental changes in the skull. Among mammals and non-mammalian amniotes, the ossification onset of the supraoccipital bone, which covers the occipital lobe of the cerebrum and cerebellum, is more accelerated in taxa with higher EQ (Figs 3 and 4; and Table 1). This indicates that the developmental timing of the supraoccipital can predict brain size. The developmental timing of the supraoccipital is more precocious, on an average, in mammals than in non-mammalian amniotes. Furthermore, our squared-change parsimony analysis...
may be because of the pleiotropic effect of Lmx1b. The link between supraoccipital development and brain expansion is not significantly different between pooled dermal bones and pooled endochondral bones (Mann–Whitney U-test, P < 0.00001). (b) Neighbour-joining cluster analysis of developmental timing. Bootstrap values were obtained through 10,000 permutations. Cranial bones form two clusters such as a dermal bone cluster and an endochondral bone cluster. (c) Topology of dermal and endochondral bones (prenatal Bos taurus). Abbreviations: as, alisphenoid; bo, basioccipital; bs, basisphenoid; de, dentary; eo, exoccipital; et, ectotympanic; fr, frontal; go, goniale; ju, jugal; la, lacrimal; mx, maxilla; na, nasal; os, orbitosphenoid; pa, parietal; pg, pterygoid; pm, premaxilla, so, supraoccipital; sq, squamosal.

Recent genetic studies have shown that the development of the supraoccipital and brain are genetically integrated. The apparent link between supraoccipital development and brain expansion may be because of the pleiotropic effect of Lmx1b and Dlx5. The supraoccipital and interparietal are either absent or severely reduced in Lmx1b knockout mice. Furthermore, this gene is critically required for mid/hindbrain development. Dlx5 is essential for axonogenesis and nervous system development and is reported to be related to Down Syndrome in humans. It also affects the timing of supraoccipital ossification, and more importantly, Dlx5 null mutants explicitly lack the supraoccipital and interparietal.

Our modularity analysis demonstrates that timing of ossification of dermal bones is constrained towards earlier developmental stages, whereas that of endochondral bones occurs later. Furthermore, the skull bones form two separate modules, one consisting explicitly of dermal bones and the other of endochondral bones. It is possible that when sequence heterochrony occurs during evolution, developmental timing of bones of identical developmental modes are likely to covary, and that bones of different developmental modes tend to be more independent from each other. On the other hand, neither mesoderm versus neural crest origin nor phenotypic modularity identified based on adult metric traits appears to be related to cranial ossification heterochrony. In the early stages of vertebrate development, Hedgehog signalling critically controls the differentiation of osteoblasts and the onset of osteoclast activity in endochondral bones, while alteration of this signalling has little effect on dermal bone formation. Such a finding implies the
genetic independence of endochondral bones and dermal bones, that is, timing of osteogenesis of endochondral bones and dermal bones are controlled by somewhat independent gene regulatory networks. Together, we suggest that such genetic integration constrains cranial ossification timing both ontogenetically and evolutionarily.

Our study highlights the conserved modular organization imposed on cranial heterochrony and the evolutionary degrees of freedom in this integrated system. Ossification modes fundamentally constrain the evolvability of cranial development. Although the integration of all other endochondral bones is evident, the supraccoxiptal appears to be rather independent from the rest (Fig. 6b). This bone exhibits the most variable ossification timing among endochondral bones (Supplementary Table 4) and does not form a tight ossification timing cluster with other occipital elements (that is, exoccipital and basioccipital) (Fig. 6a,b), despite the shared somite derivation of all three occipital components. We suggest that this relative independence of the supraccoxiptal may be because of its tight link with the brain.

Methods

Specimen collections. Specimens were sampled at the Anthropological Institute and Museum of University of Zurich (AIMUZ), Botanical Gardens Museum of Hokkaido University (BGHU), Institute of Ecology and Biological Resource of Vietnamese Academy of Science and Technology (IEBR), Japan Monkey Center (JMC), Kyoto University Museum (KUM), Natural History Museum Bern (NMB), Natural History Museum Wien (NMW), Swedish Museum of Natural History Stockholm (NRS), National Museum of Nature and Science of Tokyo (NMST), Palaeoanthropology Institute and Museum of University of Zurich (PIMUZ), Wildlife Laboratory at Tokyo University of Agriculture (TUA), University Museum of University of Tokyo (UMUT), and Berlin Museum of Natural History (ZMB). Specimens used in this study are summarized in Supplementary Data 1.

Data acquisition. Ossification sequence data of 21 cranial elements were documented. Ectotympanic, gonial and sphenoid of mammals were homologized to angular, prearticular and epitympanic of non-mammalian amniotes, respectively. The appearance of bones was assessed non-invasively by acquiring CT80, Scanco Medical, Bassersdorf). Three-dimensional visualization of shadow images were conducted in Amira 5.3 (Visage Imaging GmbH, Berlin, Germany). Supplementary Data 2 lists the acquired sequences and those obtained from the literature.

Phylogenetic framework. The topology was arranged in Mesquite39. Phylogenetic framework of species studied and divergence time are based on molecular evidence (Supplementary Table 1). Divergence time has been estimated for all major sauropod and mammal clades. Therefore, although not completely consistent internally, the TimeTree of Life-Project40 resamples the most comprehensive synopsis of molecular-based phylogenetic studies to date. Several authors published divergence times of lower taxonomic levels. Those were brought into relation with the TimeTree of Life. Therefore, the deepest, most overlapping phylogenetic node between the TimeTree of Life-project and the specific study were compared among each other and brought into relation. The resulting factor was then used to normalize the divergence times of lower taxonomic levels in the specific study. Usually these were known only for one or two subclades. Such normalization was also performed for data of those chapters in the TimeTree of Life, which show inconsistency towards the higher phylogenetic levels of other chapters. Only few studies exist that present molecular-based divergence times of the mammalian or sauropod subgroups. Moreover, those studies often do not show nodes that overlap with nodes in the phylogeny of the TimeTree of Life or the subclades of our taxonomic sampling are not represented. In those cases, the branch lengths between the nodes of each bone were divided by the maximum divergence time internally.

Modularity analysis. Neighbour-joining cluster analysis45 based on chord distance was conducted to identify integration of bone ossification timing. Nodes were tested using bootstrapping with 10,000 permutations. Analyses were conducted with PAST46. Here again, well-resolved species only with >3 ranks were included. Then, three hypothetical module divisions, such as developmental modules (neural-crest-cell bones versus mesoderm bones), ossification mode modules (dermal bones versus endochondral bones) and phenotypic variational modules, were tested if these could be recovered in neighbour-joining cluster analysis.

Heterochrony analysis. We used two methods, squared-change parsimony23,41 and PGI24, to reconstruct heterochronic changes in amniotes. In the former approach, the sequence of each bone is divided by the maximum divergence time in intervals that are standardized between 0 and 1. Then, squared-change parsimony based on a Brownian motion model of character evolution and Felsenstein’s independent contrasts42 is used to reconstruct the heterochronic changes at all nodes. The analysis was conducted with the PDAP module of Mesquite.29 Divergence times derived from molecular dating were used as branch lengths. As the resolution of the sequence can bias the results in this approach, well-resolved species with more than three ranks were included. The alternative PGI examines the sequence as one single, complex character and uses the Parsimov algorithm as an edit-cost function to optimize ancestral states and sequence heterochronies. The PGI algorithm computes the lowest cost assignment of the ancestral sequences in a two-step, dynamic programming procedure. The advantage of this approach is that no assumptions are made of the data, outside of those made when evaluating the hypothetical solutions. The parameters used for the analysis were as follows: 100 cycles, 100 replicates and 100 sequences retained at each node. Semi-exhaustive search with 10,000 permutations was performed. Such runs were conducted four times independently, and the shortest tree was treated as the conservative reconstruction. As the phylogenetic position of turtles is still disputed, and as results by PGI can be affected by polytomy, turtles were excluded from this analysis.

The analysis was conducted using ‘ape’, ‘e1071’, and ‘PGI’ packages in R24

Comparisons with brain size. We compared the relative timing of cranial ossification (scaled from 0 to 1) and EQ26. Phylogenetic effect was corrected using Felsenstein’s independent contrasts. Significance level was set as P = 0.05/21 after Bonferroni correction. Species with <3 ranks were excluded from this analysis to minimize statistical errors. EQ for mammals was calculated following the alternative formula (Log10(body weight) − Log10(bra) − Log10(bra) × 0.515) reported by Boddy et al.31, and EQ for non-mammalian amniotes (Log10(body weight) − Log10(body weight × 0.515) were computed following the formula reported by Witmer et al.20

Analysis of variation in ossification sequence. To examine the rank variation in sequence of a particular ossification event, we scaled the rank of each ossification event as:

\[ r \text{ in which } r \text{ is the absolute rank of a given ossification event, and } r_{\text{max}} \text{ is the total number of ranks for each species. Therefore, the relative ranks of each species are distributed between 0 and 1. This allows removing the differences of maximum ranks between species resulting in weighting levels of sampling that are absolute between species. A similar approach as standardizing the absolute rank } r \text{ by the maximum number of ranks } (r_{\text{max}}) \text{ has been applied in previous sequence heterochrony studies. The ranks are distributed between 1/r_{\text{max}} and 1 with this method, the relative ranks of the earliest bone to ossify can vary, depending on } r_{\text{max}}. \text{ However, the method used here circumvents this problem because the relative ranks of the earliest event is always scaled to zero. Nevertheless, some noise remains because species with higher } r_{\text{max}} \text{ have a lower influence on the variance. The range in rank variation across species was assessed to examine the variability of a particular element in the ossification sequence. As the resolution of the sequence can bias the results in this approach, species only with >3 ranks were included.}

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