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Conserved relative timing of cranial ossification patterns in early mammalian evolution

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SUMMARY We analyzed a comprehensive data set of ossification sequences including seven marsupial, 13 placental and seven sauropsid species. Data are provided for the first time for two major mammalian clades, Chiroptera and Soricidae, and for two rodent species; the published sequences of three species were improved with additional sampling. The relative timing of the onset of ossification in 17 cranial elements was recorded, resulting in 136 event pairs, which were treated as characters for each species. Half of these characters are constant across all taxa, 30% are variable but phylogenetically uninformative, and 19% potentially deliver diagnostic features for clades of two or more taxa. Using the conservative estimate of heterochronic changes provided by the program Parsimov, only a few heterochronies were found to diagnose mammals, marsupials, or placentals. A later onset of ossification of the pterygoid with respect to six other cranial bones characterizes therian mammals. This result may relate to the relatively small size of this bone in this clade. One change in relative onset of ossification is hypothesized as a potential human autapomorphy in the context of the sampling made: the earlier onset of the ossification of the periotic with respect to the lacrimal and to three basicranial bones. Using the standard error of scaled ranks across all species as a measure of each element's lability in developmental timing, we found that ossification of early, middle, and late events are similarly labile, with basicranial traits the most labile in timing of onset of ossification. Despite marsupials and placental mammals diverging at least 130 Ma, few heterochronic shifts in cranial ossification diagnose these clades.

INTRODUCTION

The rich ecomorphological diversity of mammals involves adaptations to live in very diverse kinds of habitats and a wide palette of locomotory and sensory specializations. These are reflected in a skull, which varies tremendously in its proportions and shape across clades (e.g., Fig. 1). The mammalian skull is one of the best studied vertebrate anatomical systems in its evolution and function (Starck 1995). However, very little is known about the timing of development of its parts. A main factor which contributed to this is the difficulty of collecting mammalian developmental series. Here, we provide new cranial ossification data in several nonmodel organisms and use recently developed analytical tools to study these and previously published data in a comprehensive assessment of evolutionary changes in developmental timing.

Central in studies of mammalian skeletal heterochrony is the examination of the marsupial–placental dichotomy. These two groups possess fundamentally different reproductive and life-history strategies. Marsupial young are born after an extremely short intrauterine period and are characterized by a short time of organogenesis. In contrast to placentals, most maternal investment in marsupials occurs during an extended postnatal period via lactation. The relative timing of development of craniofacial and limb structures in marsupials is probably affected by functional requirements associated with their reproductive mode (Smith 1997; Maier 1999; Sears 2004; Weisbecker et al. 2008).

In a landmark paper, Smith (1997) provided the first comprehensive study of cranial heterochronies in mammals using the "event-pairing" method based on the study in nine mammalian species of 12 bones and several other musculo-skeletal and neural elements, expanded by Nunn and Smith (1998) to 10 mammal species in a statistical examination of heterochrony. These authors concluded that the central nervous system (CNS) of marsupials is delayed in its development relative

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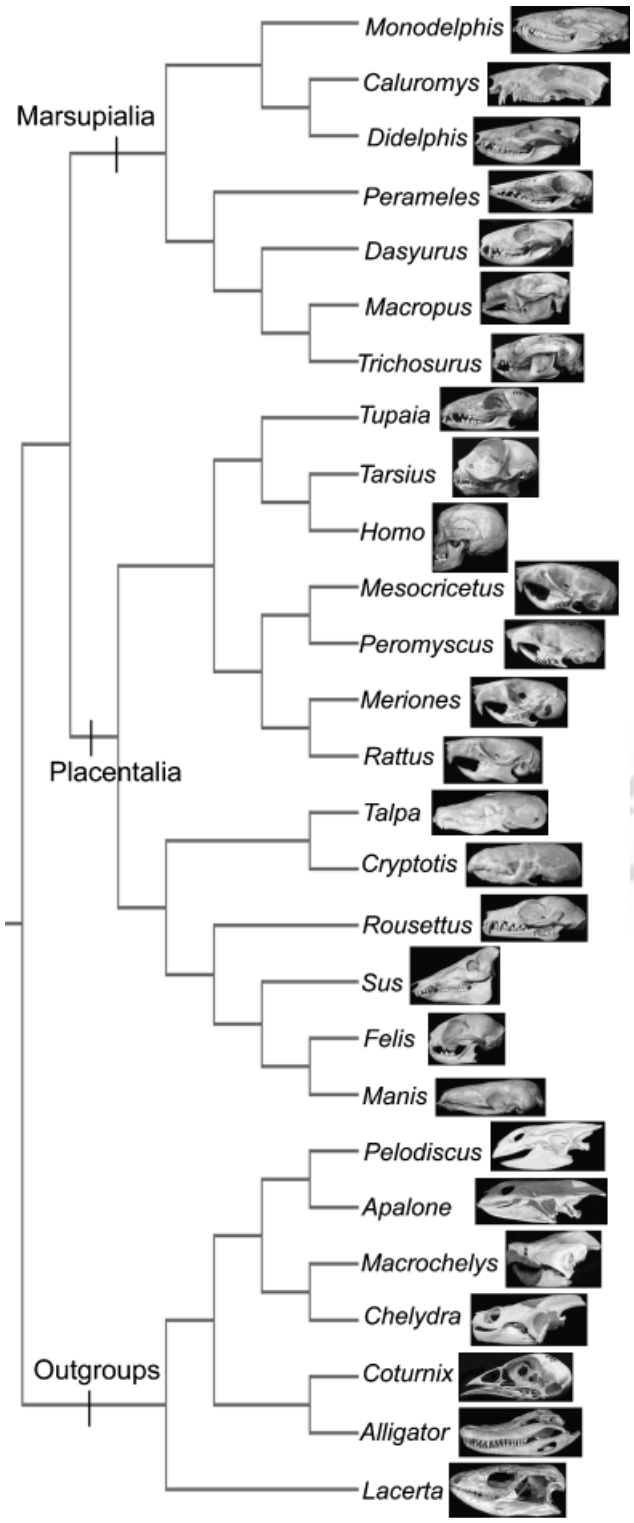

Fig. 1. The phylogenetic framework on which the sequence data were examined and taxonomic names mentioned in the text. See "Materials and Methods" for sources used. Skull images based on museum specimens in Zürich and Cambridge University Museums.

to other cranial structures or that the CNS is advanced in placental mammals, and discussed other potential heterochronies in the head region (see also Jeffery et al. 2002). By increasing the taxon sampling to twice of what was previously studied (Nunn and Smith 1998) and by considering a more robust phylogenetic framework for the mammals studied, as well as the outgroup taxa, we provide a reliable quantification of heterochronic shifts in cranial skeletal development in mammals. We use the recently developed Parsimov method (Jeffery et al. 2005), which is based on parsimony and provides the minimal solution that accounts best for the changes in relative developmental timing. With this, we provide the most comprehensive sampling and rigorous analysis of mammalian cranial heterochrony to date.

The role of heterochrony as a major evolutionary mechanism is contested (Raff 1996; Klingenberg 1998; Richardson 1999), and few studies have attempted to quantify it. The most comprehensive study of sequence heterochrony in mammals is Bininda-Emonds et al. (2003a). These authors analyzed 116 organogenetic events in 20 mammalian species based on data from old and neglected embryological literature. They concluded that besides some differences between placentals and marsupials (of the latter, data for just the opossum were available), there were few heterochronic changes in the early embryonic period. This work left several questions open: How common is heterochrony in later events in ontogeny (e.g., ossification)? How does sampling of entirely unexamined clades in marsupials and placentals affect the interpretation of heterochrony?

MATERIALS AND METHODS

Recording of ossification sequences and taxa studied

The onset of ossification in 17 elements of the skull was recorded for seven marsupial, 13 placental and seven species comprising a monophyletic outgroup. The phylogenetic framework on which the data were examined is a composite of several sources (Fig. 1). Interordinal relationships among marsupials followed the congruent topology suggested by the following studies: the combined morphological–molecular analysis of Asher et al. (2004), the super tree of Cardillo et al. (2004), and the analysis of the original data set of mitochondrial and nuclear gene data of Phillips et al. (2006). Interordinal placental relationships follow Springer et al. (2004). Relationships among rodents follow Steppan et al. (2004). Relationships among members of the outgroup follow Mickoleit (2004). Although the traditional view has been for a basal position of turtles among amniotes, more recent studies reject this hypothesis (Rieppel and de Braga 1996; Zardoya and Meyer 2001). Molecular (e.g., Zardoya and Meyer 2001) and ongoing studies of various integumentary character complexes (e.g., Scheyer 2007) favor archosaurs as the living sister-group of turtles. The relationships among the four turtle taxa considered in the analysis are uncontroversial (Gaffney and Meylan 1988).

Making the outgroup paraphyletic by adding at least one lissamphibian species could potentially add relevant resolution for polarity issues in this kind of study, and this is a potential avenue of research for the future. However, increasing outgroup sampling outside Sauropsida would also add problems of homologization of skull structures, as is characteristic of large-scale phylogenetic studies (Rieppel 2007). With this in mind, we assume in this study that the sampling of several sauropsids provides a reasonable hypothesis for the last common ancestor of that clade, which is compared with mammals as sampled here. The diagnostic features of mammals found in this work are then recognized as potential autapomorphies to be tested in future, enlarged analyses.

Here, we present for the first time data on cranial ossification sequences for a bat (*Rousettus amplexicaudatus*), for a shrew (*Cryptotis parva*), and for two rodent species (*Peromyscus melanophrys*, *Meriones unguiculatus*). Furthermore, the resolution of the sequence data for several marsupial and some placental species is improved compared with that presented in previous publications (Nunn and Smith 1998; Goswami 2007) through the examination and documentation of additional specimens. Table 1 lists the sources of materials for the data analyzed. The mammalian taxa examined comprise a spectrum of ecomorphological adaptations and a phylogenetic breadth that includes representatives of most major groups of marsupials and of Laurasiatheria (pig, cat, pangolin, fruit bat, shrew, and mole) and Euarchontoglires (rodents, tree shrew, and primates) among placentals. Afrotheria (e.g., elephants, tenrecs, and sea cows) and Xenarthra (e.g., anteaters) are not represented in the sample. Published data on sauropsids were used as outgroups. There are published data for a few other species, but these were not included in the analysis because of lack of resolution in the reported sequences or a large proportion of missing data for the cranial elements considered in our study.

Table 1. Summary of the new, published, and expanded used in the analysis of sequence of onset of ossification

Taxon	Common name	# of specimens	Ordering criterion	References
Monodelphis domestica	Gray short-tailed opossum	28	Age	Nunn and Smith (1998), Goswami (2007)
Caluromys philander	Bare-tailed woolly opossum	9	Size	Goswami (2007), this work
Didelphis albiventris	White-eared opossum	16	Size	Oliveira et al. (1998)
Perameles nasuta	Long-nosed bandicoot	10	Size	Nunn and Smith (1998), Goswami (2007)
Dasyurus viverrinus	Eastern quoll	18	Size/ stage/age	Nunn and Smith (1998), Goswami (2007)
Macropus eugenii	Tammar wallaby	20	Size/ stages/ ages	Nunn and Smith (1998)
Trichosurus vulpecula	Common brushtail possum	6	Size	Goswami (2007), this work
Tupaia javanica	Horsfield's treeshrew	24	Stage	Nunn and Smith (1998), Zeller (1987), Goswami (2007)
Tarsius spectrum	Spectral tarsier	21	Stage	Nunn and Smith (1998)
Homo sapiens	Human	60	Size	Mall (1906), Davies and Parsons (1927)
Mesocricetus auratus	Golden Hamster	168	Age	Beyerlein et al. (1951), Kanazawa and Mochizuki (1974)
Peromyscus melanophrys	Plateau mouse	13	Age	This work
Meriones unguiculatus	Mongolian gerbil	9	Age	This work (S. Kuratani, unpublished data)
Rattus norvegicus	Norway rat	Not specified, 14 stages	Age	Strong (1925)
Talpa spp.	European moles	16		Goswami and Prochel (2007), Prochel et al. (2008), this work
Cryptotis parva	Least shrew	15	Age	This work
Rousettus amplexicaudatus	Geoffroy's rousette fruit bat	11	Size	This work
Sus scrofa	Feral pig	10	Size/stage	Nunn and Smith (1998)
Felis domestica	Cat	17	Size/age	Nunn and Smith (1998)
Manis javanica	Sunda pangolin	12	Size/stage	Nunn and Smith (1998)
Pelodiscus sinensis	Chinese soft-shelled turtle	49	Age/stage	Müller et al. (2007), submitted
Apalone spinifera	Spiny soft-shelled turtle	40	Stage	Sheil (2003)
Macrochelys temminckii	Alligator snapping turtle	22	Stage	Sheil (2005)
Chelydra serpentina	Common snapping turtle	47	Stage/size	Rieppel (1993a), Sheil and Greenbaum (2005)
Coturnix coturnix	Common quail	15	Age	Nakane and Tsudzuki (1999)
Alligator mississippiensis	American alligator	36	Age/stage	Rieppel (1993b)
Lacerta vivipara	Common lizard	23	Size	Rieppel (1992)

To prepare the specimens, we used a modified version of the standard enzymatic clearing and double staining (Prochel 2006). The earliest sign of ossification was recorded based on uptake of alizarin red (Fig. 2). Some of the data gathered from published reports were obtained using stained histological sections. Detection of the onset of ossification can be slightly earlier or later depending on the method used (Vogel 1972, p. 1282). This is not a source of error in our analysis because the same method was consistently used within a species.

Because missing data are a common problem in event-pair analysis, for example leading potentially to spurious reconstructions of evolutionary change, elements that never ossify were coded as the last ones to do so, instead of as "missing." For example, *C. parva* does not have a jugal, so this was coded as the last element in the sequence to ossify for this species. In the turtles, the nasal and lacrimal are coded as last elements, as these two bones are absent in these species. In all outgroups except for the Japanese quail (Nakane and Tsudzuki 1999), the orbitosphenoid is coded as last element, given its absence. Sidor (2001) considered the orbitosphenoid a neomorph of Mammalia, although it should be noted that a bone of the same name (probably not homologous) is identified in other tetrapods (e.g., Bellairs and Gans 1983).

Data for *Homo sapiens* were based on Mall (1906), with additions from Davies and Parsons (1927) as summarized by Johnson (1933). Data for *Talpa* spp. are based on *Talpa europaea* (Goswami and Prochel 2007), with two minor additions to add resolution toward the end of the sequence based on *Talpa occidentalis* (Prochel et al. 2008), resulting in a "chimera." We think this alternative is better than leaving moles out of the analysis, which would happen if we were to treat the two species separately. The published data for these moles show that their ossification sequences are congruent as to justify our procedure. The data for *Mesocricetus auratus* from Kanazawa and Mochizuki (1974, table 5) were the "earliest appearance" time, as opposed to the mean values reported in the same table 5 by these authors.

Oliveira et al. (1998) referred to the "incisive" facial bone, what we assume to be the premaxilla. Based on comparative and/or unpublished data, the early ossification of the dentary in the following taxa was assumed: *Perameles*, *Dasyurus*, *Macropus*, *Trichosurus*, *Tarsius*, *Felis*, *Sus*, and *Manis* (Table 2).

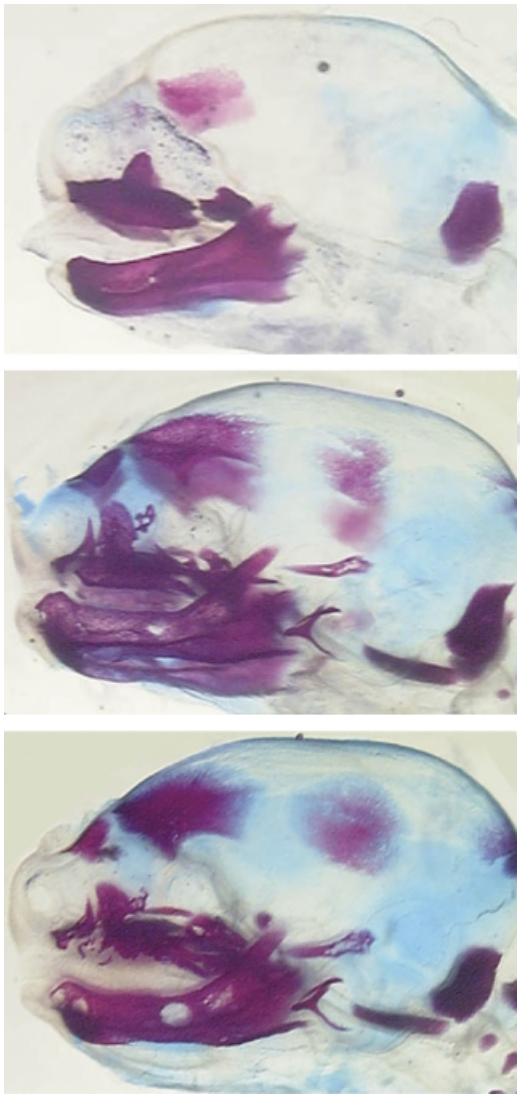

Fig. 2. Examples of cleared and stained heads at different stages of development. Postnatal days 1, 4, and 6 (top to bottom) in the opossum *Monodelphis domestica*. Not to scale.

Event pairing

For all species a matrix was constructed in which the onset of ossification in the 17 elements was compared with every other event. These resulted in 136 event pairs (characters) for each species (Smith 1997; Jeffery et al. 2002). Three character states reflect the relative timing of one event relative to another: before (2), simultaneous (1), or after (0). Simultaneous events are usually the result of incomplete sampling, because it is unlikely that the onset of ossification of two bones occurs exactly at the same time (Nunn and Smith 1998, p. 87).

In addition to mapping the event pairs on the phylogeny in order to document patterns of change in them (Smith 1997), we used Parsimov (Jeffery et al. 2005) to analyze the sequence data. This method determines the minimal number of heterochronic events that accounts for every event-pair change and yields a consensus that contains all hypotheses of movement that must necessarily form part of any equally most parsimonious solution to the observed event-pair changes (Jeffery et al. 2005, p. 239). It provides a very conservative estimate of change, in contrast with the simple mapping method or the more subjective cracking method (Jeffery et al. 2002). The alternative accelerated transformation (ACCTRAN) or delayed transformation (DELTRAN) optimizations, also reported here, have more heterochronies reported than the more conservative consensus Parsimov output. In the case of ambiguous reconstruction of evolutionary change, the ACCTRAN option provides "accelerated transformations", and with that favors reversals on the reconstructed pattern of change. The DELTRAN option instead, favors convergences (Maddison and Maddison

Table 2. Relative timing of onset of ossification (ranks) in the elements and taxa analyzed in this study, the raw data for the event-pairing analysis

	Monodelphis	*Caluromys*	*Didelphis*	*Perameles*	*Dasyurus*	*Macropus*	*Trichosurus*	*Tupaia*	*Tarsius*	*Homo*	*Talpa.*	*Cryptotis*	*Peromyscus*	*Rousettus*	*Meriones*	*Rattus*	*Mesocricetus*	*Felis*	*Sus*	*Mamis*	*Pelodiscus*	*Apalone*	*Chelydra*	*Macrochelys*	*Alligator*	*Lacerta*	*Coturnix*
Premaxilla	1	1	1	1	1	1	1	1	1	2	1	1	1	3	1	2	2	1	1	1	5	6	2	3	1	1	2
Maxilla	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	2
Palatine	3	3	2	3	3	?	3	4	2	4	1	2	2	3	3	2	2	?	?	?	3	3	1	4	3	1	2
Dentary	1	1	2	?	?	?	?	1	?	1	1	1	1	1	1	1	?	?	?	?	1	2	1	2	1	1	1
Frontal	2	2	3	3	2	2	2	2	2	3	1	2	2	2	1	1	2	2	1	2	4	3	2	4	2	2	2
Parietal	4	2	3	5	4	3	4	2	3	3	1	3	2	2	4	2	2	3	3	2	2	3	2	4	5	2	3
Squamosal	3	3	6	4	4	2	4	2	2	3	1	5	4	1	4	2	2	4	1	2	2	1	1	2	2	2	1
Basioccipital	4	4	3	6	5	4	4	4	6	6	1	6	2	4	2	2	3	5	5	3	5	5	5	5	6	4	3
Nasal	4	3	5	4	5	?	3	4	?	4	1	4	4	3	3	3	?	?	?	?	8	8	?	?	4	2	1
Pterygoid	7	5	3	5	5	?	3	5	?	4	1	3	2	4	2	2	?	?	?	?	3	3	1	2	1	1	1
Exoccipital	2	3	3	3	3	2	3	3	4	3	2	6	3	4	3	4	2	5	3	3	6	5	4	4	3	3	3
Basisphenoid	6	4	3	8	6	5	4	6	6	8	2	7	3	5	4	4	2	6	6	4	4	5	4	4	3	4	3
Jugal	3	2	3	2	3	2	2	2	2	3	3	11	4	2	4	3	5	3	4	1	3	4	3	4	1	2	1
Lacrimal	3	4	4	6	5	?	3	4	3	7	4	6	4	3	4	3	6	?	?	?	8	8	?	?	2	5	?
Alisphenoid	5	3	?	5	4	3	3	4	5	5	6	8	4	5	4	5	4	5	3	3	7	7	6	6	?	3	?
Orbitosphenoid	7	5	?	7	6	?	4	5	?	7	7	10	5	7	5	6	7	?	?	?	8	8	7	7	7	6	4
Periotic	8	6	?	9	7	6	4	7	7	4	5	9	5	6	?	?	?	7	7	4	6	5	5	5	6	4	4

1992). The consensus of the two, reconstructs the common non-ambiguous reconstructions, and with that less changes.

To provide a quantitative measure of the relative lability of elements, we scaled the rank of each by the total number of ranks for each species. The standard error of scaled element ranks across all species was used as a measure of each element's lability in developmental timing. Although more sophisticated measures are available (Poe 2006), multiple simultaneous events in all of the taxa make the application of such methods problematic. The one used here is comparable to that used in Bininda-Emonds et al. (2003b) in providing a measure of the standard error for each element, and a comparison of the standard error of rank against median rank. We use minimum rank in addition to median rank, because a question of particular interest is whether early occurring events are less labile than later events, and using median rank may hide highly labile events that occur early in the sequence only occasionally. Nonetheless, these occasionally early events still can demonstrate that the early sequence is labile, and for this reason, we include analyses using both median and minimum ranks. Because *Mus* and *Talpa* have particularly low resolution, they were removed from the data set before the analysis of element lability.

Finally, as an empirical test of the use of event-pairing data in phylogenetic reconstruction, a parsimony analysis (heuristic search) of the 136 "characters" described above was conducted using PAUP* version 4.0b10, using default heuristic search settings (Swofford 2001).

RESULTS

A total of 22 event pairs exhibit the same state for all taxa (ingroup and outgroup) examined, meaning that 22 pairs of bones have the same relative timing across all species, and 25 are invariable except for "ties" (i.e., simultaneity as coded by state "1"). This makes a total of 47 (34.6%) of "uninformative" event pairs. Twenty-two event pairs are invariable (exhibiting the same state or ties) within all mammals, bringing the total number of uninformative event pairs to 69 (50.7% of total). Of these invariable 22 event pairs, one clearly differentiates mammals from the outgroups and is potentially an autapomorphy: the onset of ossification of the pterygoid after the premaxilla. The DELTRAN optimization in Parsimov shows for marsupials a late ossification of the pterygoid, relative to the palatine, squamosal, and nasal.

Forty-one (30.1%) event pairs are autapomorphies or patterns within mammals that do not provide any phylogenetic signal. Of these, four event pairs provide a potential human autapomorphy (we cannot discount the possibility that it is a synapomorphy of Anthropoidea or any less-inclusive clade within Anthropoidea that includes humans): the earlier onset of ossification of the periotic with respect to the lacrimal, basisphenoid, basioccipital, and alisphenoid elements (the latter also the case in *Talpa* spp.)

Table 3. List of the movements in the onset of ossification of cranial elements for the major groups in the phylogeny presented in Fig. 1, as reconstructed by the Parsimov method

Basal node—mammals
DELTRAN
Pterygoid moved late relative to Premaxilla, Maxilla, Dentary, Parietal
ACCTRAN
Pterygoid moved late relative to Maxilla, Palatine, Dentary, Parietal, Squamosal, Nasal
CONSENSUS
Pterygoid moved late relative to Maxilla, Dentary, Parietal
Basal node—outgroups
DELTRAN
Twins (Palatine, Exoccipital, Pterygoid, Jugal)
ACCTRAN
Twins (Basisphenoid, Lacrimal)
Palatine moved E relative to Frontal, Exoccipital
CONSENSUS
No movement
Basal node—placentals
ACCTRAN
Parietal moved early relative to Squamosal, Jugal
DELTRAN
Twins (Parietal, Jugal)
CONSENSUS
No movement
Basal node—marsupials
DELTRAN
Twins (Orbitosphenoid, Periotic)
Pterygoid moved late relative to Palatine, Squamosal, Nasal
ACCTRAN
Twins (Lacrimal, Basioccipital, Orbitosphenoid, Periotic)
CONSENSUS
Twins (Orbitosphenoid, Periotic)

Transformations occurring for other groupings are listed in Appendix A. As the different outgroups used form a monophyletic clade, additionally more basal outgroups would be needed to root the tree and test the movements at the base of the tree as potential autapomorphies of mammals or sauropsids.
ACCTAN, accelerated transformations; DELTRAN, delayed transformations.

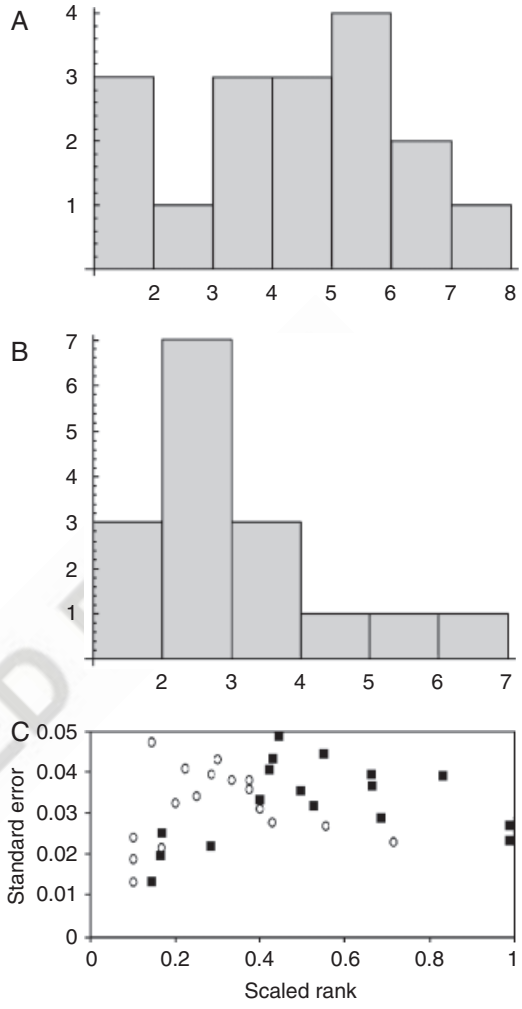

Fig. 3. Rank variability. Histograms of element ranks in (A) a well-resolved taxon, *Dasyurus*, and (B) a skewed data set, *Rattus*, displaying positive skewness due to more early events recorded than late events. (C) plot of scaled median rank (closed squares) or scaled minimum rank (open circles) of elements against standard error of ranks, showing the middle rank elements have higher variability, but that many early events also show high variability.

Table 3 presents a list of the movements in the onset of ossification of cranial elements in the major groups examined (Fig. 1) as reconstructed by Parsimov. Additional transformations for other groups are listed in Appendix A.

The results of the rank variability analyses are presented in Fig. 3. There is a relatively even distribution of events in most taxa; only *Didelphis*, *Talpa*, and *Rattus* are significantly kurtosed (Fig. 3A). Event distributions of many taxa are weakly and positively skewed (Fig. 3B), suggesting a greater concentration of ossification events and/or less resolution early in the sequence. Measures of rank variability show that elements with early to middle ranks show the highest variability when minimum ranks are used, whereas use of median ranks show the highest variability in events occurring in the middle of the sequence (Fig. 3C). The most variable elements in developmental ranks are primarily basicranial or zygomatic, with the squamosal, pterygoid, jugal, parietal, basioccipital, and the basisphenoid the most variable. The least variable elements are primarily anterior, including the dentary, maxilla, frontal, orbitosphenoid, and the premaxilla.

Figure 4 shows the consensus of four equally parsimonious trees (length, 517 steps) resulting from the parsimony analysis of the event-pairing data. It is highly incongruent with the well-supported phylogeny taken as reference (Fig. 1). Turtles are reconstructed in a monophyletic group, but the other

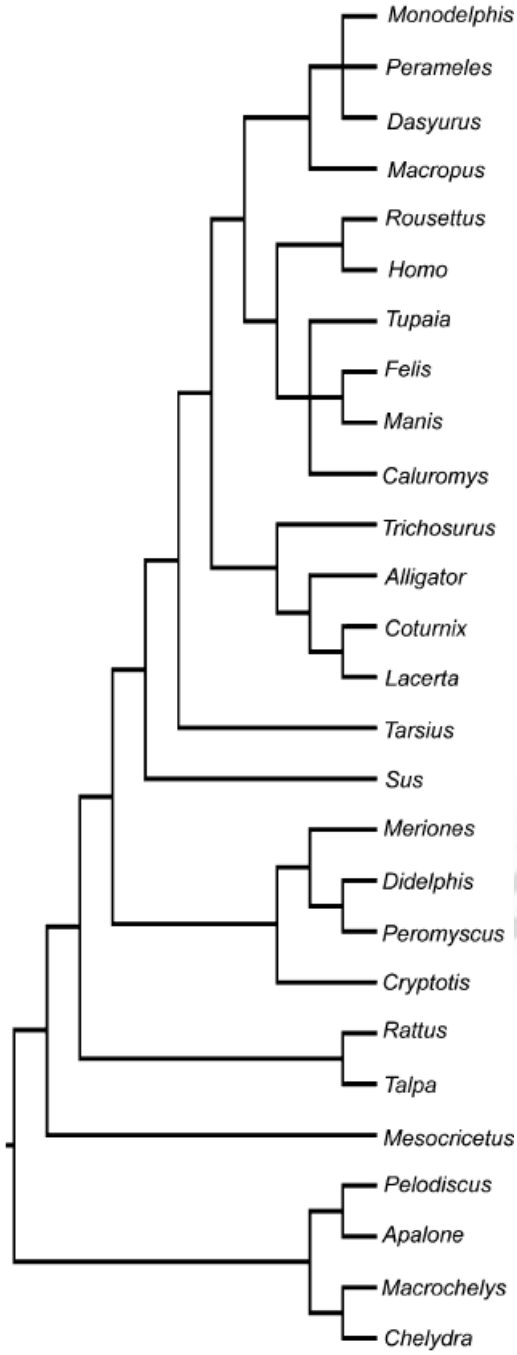

Fig. 4. Consensus tree resulting from the parsimony analysis of the 136 event pairs for 27 taxa (tree length = 520 steps). Notice the incongruence with the tree presented in Fig. 1 and the non-monophyly of all major accepted clades and of the great majority of less inclusive ones.

outgroups are clustered with a marsupial (*Trichosurus*). The opossum *Didelphis* is in a group with rodents and a shrew, and several other very unparsimonious groupings are present across the entire tree.

DISCUSSION

Shifts in the relative timing of onset of ossification have occurred in early therian mammal evolution. However, the available data suggest that sequence heterochrony is not prevalent, leading to the major clades of mammals, and not many changes within therians have occurred when compared with a sample of outgroup sauropsids (see also Bininda-Emonds et al. 2003a). Other developmental mechanisms must be associated with the generation of diverse cranial morphology among mammals. One of them could in fact be heterochrony, but of a different kind to that explored in this paper: growth heterochrony (*sensu* Smith 2001) or allometric relations among parts during growth (Klingenberg 1998; Weston 2003; Giannini et al. 2004; Cardini and Thorington 2006; Sears et al. 2007).

The little sequence heterochrony in skull elements for the basal groups of mammals examined does not mean that this phenomenon has not been important in the shaping of adult morphology in marsupials and placentals. Smith (1997) examined a more restricted taxonomic sample of mammals, but hypothesized that there is a delay in the development of the CNS elements in the case of marsupials as compared with placentals. The relative timing of brain and cranial bone development is relevant in this context. It is worth mentioning that ACCTRAN reconstruction for placentals displayed early parietal onset of ossification relative to some other cranial bones, possibly reflecting the relatively larger size of the brain of placentals in comparison with marsupials (Jerison 1973; Martin 1990).

Considering the major morphological and physiological departure of therian mammals from the last common ancestor of the outgroup taxa examined (Kemp 2005), estimated to have lived at a time around a "hard minimum" of 312.3 Ma and a "soft maximum" of 330.4 Ma (Benton and Donoghue 2007), it is remarkable as to how few changes in cranial ossification timing have occurred. Thus, we confirm Schoch's (2006) report of conservatism in cranial skeletal development in vertebrates, which was based on a more limited sample of mammals but a wider sample of other vertebrates. The skull contains elements of different embryonic origins and phylogenetic histories (de Beer 1937), and how this fact affects heterochronic change is something that could potentially be addressed in studies of modularity (Goswami 2007; Hallgrímsson et al. 2007).

The apparent higher degree of heterochrony within the sauropsids as opposed to within placental and marsupial mammals may be correlated with the longer divergence times among the species representing the major clades used as outgroups, i.e., turtles, crocodiles, and birds, than among the mammalian clades in the ingroup (Benton and Donoghue 2007). However, the sampling of sauropsids is very limited; hence, focused testing and quantification of this trend is needed.

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Based on her study of the opossum *Monodelphis domestica*, Smith (1994) concluded that the timing of cranial muscle development is not reflected in that of skeletal structures. However, it has been reported for many species and skeletal elements that muscle contractions and movements influence bone formation (Hall 2005). There is no literature on cranial muscle developmental timing at stages when cranial elements start to ossify for most of the taxa studied here. It is therefore impossible at present to hypothesize whether the sequence of ossification and its changes in evolution correspond directly to muscular activity. In this context, it is worth pointing out that little if anything is known about the mechanical stresses and factors in which the head is involved in utero (but see Rot-Nikcevic et al. 2006). One rare example of prenatal mechanical stress is found in some hystricognath rodents (belonging to the clade of the guinea-pig), which have been shown to develop tooth wear before birth (Starck 1995).

Comparisons with the limb pattern

Recent work on limb development provides a similar pattern of conservatism in early skeletal developmental timing to that of the skull reported here, in spite of significant differences in adult morphology. Bininda-Emonds et al. (2007) conducted a quantitative study of heterochrony and the origin of limb morphological diversity in tetrapods, including several mammalian species. They found that heterochronic changes in early limb development and chondrogenesis were absent within major amniote clades and that their distributions across vertebrate phylogeny are not easily correlated with adaptive phenomena related to morphological differences in the adult fore and hind limbs. For example, a bat, with its greatly enlarged forelimbs modified as wings in the adult, showed near synchrony in the early development of the fore and hind limbs, similarly to other closely related placental mammals. The case of bats is instructive and has been studied in detail by other researchers, reaching similar conclusions. Sears et al. (2006) reported that in *Carollia perspicillata* and the mouse *Mus musculus*, the digits are initially similar in development, and that subsequently those of the bat lengthen. That study demonstrated that early development bore little relation to adult morphology and that major divergences in form occurred later in development. These studies of limb development highlight the important role played by allometric or growth heterochrony in shaping adult morphology. We hypothesize that allometric growth is more important than sequence heterochrony in the development of morphological cranial novelties in mammals, a hypothesis that requires quantification.

Rank lability

Similar to the results of recent studies testing for differences in lability of developmental timing, we found the most variability occurring in elements occupying the middle ranks. However, counter to the "spinning top" model observed by Bininda-Emonds et al. (2003b) in their study, we found that elements that ossify early, in at least some taxa, were also highly variable in their developmental timing across mammals. These results are not simply due to differences in resolution of early, mid, and late ossification, but, along with the results of the Parsimov analyses, instead suggest that developmental timing is more labile, and likely less integrated (Schoch 2006; Goswami 2007) than previously thought. It is also noteworthy that facial elements were observed to be less labile than neurocranial, particularly basicranial, traits.

Event-pairing approaches and methodological issues

The points raised above are well supported by the data in this study, but the nature of Parsimov, the method of heterochrony identification used here, should not be ignored. We think that the Parsimov approach is best characterized as conservative. The Parsimov analysis identified relatively few changes diagnosing the major groups of mammals examined. An example is the case of marsupials. From the mapped characters, it appears that the accelerated onset of ossification of the exoccipital bones (Fig. 2) in relation to the squamosal and the pterygoid and the delayed onset of ossification of the periotic relative to the orbitosphenoid are marsupial autapomorphies. However, none of these appear as diagnostic under the Parsimov method. In the branch leading to marsupials, Parsimov identified the "twins" orbitosphenoid–periotic. These are interpreted as events that are apparently, but not actively, moving (Jeffery et al. 2002, pp. 482, 485, 490). In addition to looking at the consensus results of the Parsimov analysis, we recommend examining simple character mapping in an exploration of potential heterochronies.

A late development of the pterygoid relative to several other bones characterizes mammals. In crown mammals, the pterygoid is a relatively small bone after a reduction from the condition of early synapsids (Sidor 2001), whereas in the outgroup taxa examined, it is larger relative to other cranial elements (Romer 1956; Gaffney 1979). This fits the hypothesis of Huxley (1932), who suggested that the time of initiation of an organ in the embryo is related to its subsequent size in the adult. Accordingly, structures that grow to be relatively large will begin developing relatively early in comparison with smaller structures ("predisplacement").

The homologization of cranial elements across mammalian taxa with the diverse outgroup taxa considered in this study, takes a standard approach that most comparative anatomists would accept. However, this approach belies the diversity and complexity in the development of several cranial elements across amniotes. For example, the pterygoid of mammals and of reptiles are complex bones in comparison with their

vertebrate ancestors, and it is unclear as to what extent the adult element (or parts of adult elements) are homologous (de Beer 1937). The paleontological record is too incomplete to provide resolution to this issue. The embryological record is also problematic, due to variation in number of ossification centers in developing bones that are presumed to be homologous in adults. Future comparative ontogenetic work, both qualitative and quantitative, will hopefully provide further tests of homology and documentation of the developmental plasticity of mammals in time and in space. The effect that alternative criteria for homologization would have on studies of heterochrony like the one performed here is unknown. One alternative criterion could be based on centers of ossification, which may not be a desirable approach to take as it would require more assumptions than the standard topological criteria based on adult specimens. What becomes obvious is that the more taxonomically inclusive a study of this kind becomes, the more difficult the homologization of structures become. This situation for large-scale cladistic morphological analyses is discussed at length and using vertebrate examples by Rieppel (2007).

Our study provides a further example of the inadequacy of using event-pairing data as characters for phylogenetic reconstruction (Sánchez-Villagra 2002; Schoch 2006). The methodological problems involved in this process (Schulmeister and Wheeler 2004), including nonindependence and spurious ancestral state reconstructions, argue against the use of these kinds of data. However, we do not think that convergences are more or less of a problem with this data partition (heterochronic data for a particular character complex of the skeleton) than with any other kind of phylogenetic study. Although both functional aspects and "constraints" in all their forms potentially affect the relative timing of developmental events, these factors also influence all kinds of other data (including molecular, Lee 1999).

Outlook into quantitative comparative ontogeny

The newly available analytical tools to examine sequence heterochrony and a rapidly improving phylogenetic framework in which to examine developmental changes set the stage for taxonomically comprehensive studies of the kind presented here. The quantification of developmental patterns is still in its infancy, a point often missed because of the long and illustrious history of comparative anatomy (Asher et al. 2008). Our empirical study suggests that sequence heterochrony in early skull ossification is not prevalent when examining most of the major groups of therian mammals and that sequence heterochrony data do not constitute a reliable phylogenetic marker. Although these results for skull heterochronies in mammals may or may not be generalizable to other taxa or organ systems, it is clear that more empirical work is needed to understand the relation between heterochrony and evolutionary patterns.

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APPENDIX A

Table A1. Summary of ACCTRAN and DELTRAN transformations as estimated by PARSIMOV leading to clades of two or more taxa, excluding those for mammals, outgroups, placentals, and marsupials, listed in Table 1 of the main text

ACCTRAN transformations
– *Monodelphis*+(*Caluromys*+*Didelphis*)
Twins (Squamosal, Palatine)
Pterygoid moved L relative to Basisphenoid, Orbitosphenoid
– *Caluromys*+*Didelphis*
Parietal moved E relative to Exoccipital, Jugal
Basisphenoid moved E relative to Basioccipital, Lacrimal
Alisphenoid moved E relative to Palatine, Nasal, Exoccipital
– Australidelphia
Twins (Orbitosphenoid, Basisphenoid)
Parietal moved L relative to Nasal, Alisphenoid
– *Dasyurus*+ (*Macropus*+*Trichosurus*)
Twins (Basisphenoid, Orbitosphenoid)
Nasal moved L relative to Pterygoid, Alisphenoid
– (*Tupaia*+[*Tarsius*+*Homo*]\ + Rodentia
Twins (Exoccipital, Squamosal)
Palatine moved L relative to Basioccipital, Pterygoid
– *Tupaia* (*Tarsius*+*Homo*)
Twins (Jugal, Frontal) (Orbitosphenoid, Basisphenoid)
Basioccipital moved L relative to Pterygoid, Alisphenoid
– *Tarsius*+*Homo*
Twins (Palatine, Basioccipital) (Dentary, Premaxilla) (Squamosal, Frontal)
Lacrimal moved L relative to Nasal, Alisphenoid, Orbitosphenoid
– Rodentia
Twins (Orbitosphenoid, Periotic)
Premaxilla moved L relative to Maxilla, Frontal
Jugal moved L relative to Parietal, Nasal, Lacrimal, Alisphenoid
– *Peromyscus*+*Mesocricetus*
Frontal moved L relative to Palatine, Pterygoid
Basisphenoid moved E relative to Nasal, Exoccipital, Jugal, Lacrimal
– *Meriones*+*Rattus*
Frontal moved E relative to Maxilla, Dentary, Parietal
– Laurasiatheria
Twins (Lacrimal, Alisphenoid)
Premaxilla moved L relative to Palatine, Nasal
Squamosal moved E relative to Dentary, Jugal
Exoccipital moved L relative to Palatine, Basioccipital, Nasal
– *Talpa*+*Cryptotis*
Palatine moved E relative to Frontal, Parietal
Nasal moved E relative to Squamosal, Lacrimal
Pterygoid moved E relative to Parietal, Squamosal, Nasal
Jugal moved L relative to Parietal, Basioccipital, Exoccipital
– *Rousettus*+ (*Sus* [*Felis*+*Manis*])
Dentary moved E relative to Premaxilla, Maxilla
Squamosal moved E relative to Palatine, Frontal, Parietal
Lacrimal moved E relative to Premaxilla, Palatine, Basioccipital, Exoccipital
– *Sus* (*Felis*+*Manis*)
Twins (Frontal, Parietal)
Alisphenoid moved E relative to Basioccipital, Exoccipital
– *Felis*+*Manis*
Twins (Basioccipital, Alisphenoid)
Squamosal moved L relative to Frontal, Parietal, Jugal
– ([*Pelodiscus*+*Apalone*]+[*Chelydra*+*Macrochelys*]) + (*Coturnix*+*Alligator*)
Premaxilla moved L relative to Dentary, Jugal
Squamosal moved E relative to Palatine, Pterygoid
Nasal moved L relative to Alisphenoid
Alisphenoid moved L relative to Basioccipital, Periotic
– (*Pelodiscus*+*Apalone*)+(*Chelydra*+*Macrochelys*)
Nasal moved L relative to Basioccipital, Periotic
– *Pelodiscus*+*Apalone*
Premaxilla moved L relative to Frontal, Parietal, Basioccipital, Basisphenoid
Pterygoid moved L relative to Dentary, Parietal, Squamosal
Exoccipital moved L relative to Basioccipital, Periotic
Orbitosphenoid moved L relative to Nasal, Lacrimal
– *Chelydra*+*Macrochelys*
Twins (Premaxilla, Jugal) (Squamosal, Dentary)
– *Coturnix*+*Alligator*
Parietal moved L relative to Frontal, Nasal, Exoccipital, Basisphenoid
Jugal moved E relative to Maxilla, Dentary, Frontal, Pterygoid
Lacrimal moved E relative to Frontal, Squamosal, Basioccipital, Nasal, Exoccipital, Basisphenoid, Periotic
DELTRAN Transformations
– *Monodelphis*+ (*Caluromys*+*Didelphis*)
Twins (Lacrimal, Nasal)
Pterygoid moved L relative to Basioccipital, Basisphenoid, Alisphenoid, Orbitosphenoid
– *Caluromys*+*Didelphis*
Twins (Basisphenoid, Basioccipital)
Parietal moved E relative to Squamosal, Jugal
Jugal moved E relative to Frontal, Squamosal
– Australidelphia
Twins (Lacrimal, Basioccipital)
Parietal moved L relative to Frontal, Exoccipital
– *Dasyurus*+ (*Macropus*+*Trichosurus*)
Twins (Orbitosphenoid, Basisphenoid)
– *Macropus*+*Trichosurus*
Frontal moved L relative to Squamosal, Jugal
– (*Tupaia*+[*Tarsius*+*Homo*])+Rodentia
Twins (Exoccipital, Squamosal)
– *Tupaia* (*Tarsius*+*Homo*)
Twins (Jugal, Frontal) (Orbitosphenoid, Basisphenoid)
Palatine moved L relative to Premaxilla, Squamosal, Nasal, Exoccipital
Pterygoid moved L relative to Squamosal, Exoccipital
– *Tarsius*+*Homo*
Twins (Squamosal, Frontal)
– Rodentia
Basioccipital moved E relative to Nasal, Pterygoid
Jugal moved L relative to Palatine, Nasal, Exoccipital
– *Peromyscus*+*Mesocricetus*
Parietal moved E relative to Palatine, Jugal

Table A1. (Contd.)

Basisphenoid moved E relative to Nasal, Exoccipital, Jugal, Lacrimal
– *Meriones*+*Rattus*
Twins (Lacrimal, Jugal)
Frontal moved E relative to Dentary, Parietal
– Laurasiatheria
Twins (Squamosal, Jugal) (Lacrimal, Alisphenoid)
Exoccipital moved L relative to Palatine, Basioccipital, Nasal
– *Talpa*+*Cryptotis*
Jugal moved L relative to Parietal, Basioccipital, Exoccipital, Basisphenoid
Alisphenoid moved L relative to Basioccipital, Basisphenoid
– *Sus* (*Felis*+*Manis*)
Twins (Frontal, Parietal) (Alisphenoid, Exoccipital)
– *Felis*+*Manis*
Twins (Basioccipital, Alisphenoid) (Jugal, Squamosal)
– ([*Pelodiscus*+*Apalone*]+[*Chelydra*+*Macrochelys*])+(*Coturnix*+*Alligator*)
Twins (Squamosal, Palatine) (Basisphenoid, Exoccipital)
– (*Pelodiscus*+*Apalone*)+(*Chelydra*+*Macrochelys*)
Premaxilla moved L relative to Maxilla, Dentary, Squamosal
Nasal moved L relative to Basioccipital, Alisphenoid, Periotic
Jugal moved L relative to Parietal, Squamosal
Alisphenoid moved L relative to Basioccipital, Periotic
– *Pelodiscus*+*Apalone*
Twins (Periotic, Exoccipital)
Premaxilla moved L relative to Frontal, Parietal, Basisphenoid, Jugal
Pterygoid moved L relative to Maxilla, Dentary, Squamosal
Orbitosphenoid moved E relative to Nasal, Lacrimal
– *Chelydra*+*Macrochelys*
Squamosal moved E relative to Dentary, Pterygoid
Orbitosphenoid moved E relative to Nasal, Lacrimal
– *Coturnix*+*Alligator*
Parietal moved L relative to Frontal, Nasal
Jugal moved E relative to Dentary, Frontal, Pterygoid

E, early; L, late; ACCTAN, accelerated transformations; DELTRAN, delayed transformations.

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