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Skeletal ossification and sequence heterochrony in xenarthran evolution

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SUMMARY Previous analyses of how mammals vary in their ossification sequences have focused on monotremes, marsupials, and boreoeutherian placentals. Here, we focus on the sequence of cranial and postcranial ossification events during growth in the xenarthran skull and skeleton, including armadillos, anteaters, and sloths. We use two different methods to quantify sequence heterochrony: sequence analysis of variance (ANOVA) and event-paring/Parsimov. Our results indicate that Parsimov is conservative and does not detect clear heterochronic shifts between xenarthran and boreoeutherian placentals. Sequence-ANOVA performs better, but both methods exhibit sensitivity to the artifactual accumulation of ties. By controlling for ties and taking into account results that the methods have in common, our analysis suggests that xenarthrans significantly differ from other placentals by a late ossification of the sternum and an early ossification of the phalanges and pubis. We interpret these differences as showing that heterochrony plays a role in the skeletal development of xenarthrans, a change from previous studies that have emphasized the developmental homogeneity of the skeleton across placental mammals.

INTRODUCTION

Recent advances in deciphering the evolutionary tree of living mammals (Murphy et al. 2007; Wildman et al. 2007; Prasad et al. 2008; Hallström and Janke 2010) have enabled the articulation of novel hypotheses regarding mammalian evolution. For example, it has become clear that the two groups with living representatives most common on southern continents—afrotherians (elephants, sea cows, hyraxes, aardvarks, sengis, golden moles, and tenrecs) and xenarthrans (sloths, armadillos, and anteaters)—comprise the first one or two branches diverging from the placental mammal Tree of Life. These two groups may form a clade (Atlantogenata), with all other placental mammals distributed in two additional sister clades (Laurasiatheria and Euarchontoglires) collectively known as Boreoeutheria. Atlantogenata and Boreoeutheria have been informally referred to as "southern" and "northern" placental mammals, respectively (Asher et al. 2009). Southern placentals appear to show a high degree of developmental distinctiveness compared to many of their northern counterparts, including late eruption of permanent teeth, nondescent of the male gonads, and in some regards a high level of vertebral variability (Sánchez-Villagra et al. 2007; Asher et al. 2009, 2011). These skeletodental features, combined with the newly recognized phylogenetic position of afrotherians and xenarthrans on the mammalian Tree of Life, raise the possibility that a developmental dichotomy between northern and southern placental mammals has been previously overlooked.

Heterochrony is the change of developmental timing and growth rates over the course of evolution (Gould 1977; Alberch et al. 1979; Smith 1997). By quantifying sequence heterochrony across marsupials and placentals, Smith (1997, 2001) demonstrated specific ways in which ontogeny distinguishes these major mammalian clades. Since then, quantitative analysis of mammalian heterochrony has further refined our understanding of the marsupial/placental dichotomy (Sánchez-Villagra 2002; Sánchez-Villagra et al. 2008; Sears 2009; Keyte and Smith 2010), and revealed a surprisingly high level of conservatism among placental mammal clades

(Bininda-Emonds et al. 2003; Goswami 2007; Weisbecker et al. 2008; Goswami et al. 2009). However, at present, the literature on mammalian sequence heterochrony has yet to include developmental series of xenarthrans. Previous studies of xenarthran development focus on the dentition (Martin 1916), skull (Schneider 1955) placentation (Benirschke 2008; Enders 2008), and the vertebral column (Hautier et al. 2010), but do not yet provide a comparative basis upon which to measure how or if southern placental mammal development departs from that of other groups.

Here, we provide novel data on xenarthran skeletal ontogeny in order to test the hypothesis that xenarthrans are developmentally distinct, focusing on cranial and postcranial ossification events during growth. We employ techniques for quantifying sequence heterochrony (Smith 2001; Goswami 2007; Sánchez-Villagra et al. 2008; Weisbecker et al. 2008) to determine the extent to which xenarthrans depart from the patterns of ossification seen in other mammals, focusing on the sequences of cranial and postcranial ossification for armadillos (*Dasypus*), sloths (*Bradypus* and *Choloepus*), and anteaters (*Tamandua* and *Cyclopes*). In tandem with data collected for other mammals (Sánchez-Villagra et al. 2008; Weisbecker et al. 2008; Wilson et al. 2010), these data will help test the generality of previous reports of developmental anomalies in xenarthrans, such as early ossification of the basicranium of sloths (Schneider 1955).

MATERIALS AND METHODS

Data collection

We sampled material from collections of the Museum für Naturkunde Berlin (ZMB), the Natural History Museum London (BMNH), the Muséum National d'Histoire Naturelle in Paris (MNHN), the Institut Royal des Sciences Naturelles de Belgiques (IRSNB), and the University Museum of Zoology Cambridge (UMZC). We incorporated data from 74 xenarthran fetuses representing five genera (Wilson and Reeder 2005): *Choloepus*, *Bradypus*, *Dasypus*, *Tamandua*, and *Cyclopes* without absolute data on individual age. Thirty-two unsexed fetuses of *Dasypus novemcinctus* were examined (Fig. 1A), ranging in size from 29 to 105 mm Crown Rump Length (CRL). We also studied 22 unsexed sloth fetuses (Fig. 1B) of both extant genera (*Bradypus* and *Choloepus*) belonging to four species: *B. tridactylus*, *B. variegatus*, *C. didactylus*, and *C. hoffmanni*. Species-level identification was based on museum identifications and cranial anatomy (Wetzel 1985) and was possible for 15 of our 22 specimens. Based on sample density, two species were used in the sequence heterochrony analysis, *B. variegatus* and *Choloepus didactylus*. The fetuses ranged from 70 to 170 mm CRL for *Bradypus* and from 120 to 200 mm CRL for *Choloepus*. In addition, we obtained images of five *Cyclopes* (*C. didactylus*) and four *Tamandua* (*T. tetradactyla* and *T.* sp.) specimens. The fetuses ranged from 41 to 73 mm CRL for *Cyclopes* and from 41 to 125 mm CRL for *Tamandua* (Fig. 1C). Species-level identification for some museum specimens was unavailable; those specimens were not used to run the analyses but helped to check the accuracy of observed sequences. Table 1 lists the sources for ossification sequences we obtained from the literature.

Three-dimensional data acquisition

Skeletons were imaged using high-resolution X-ray microtomography (μCT—Fig. 1) at the Helmholtz Zentrum (Berlin, Germany), the engineering department of the University of Cambridge (Cambridge, UK), the Natural History Museum (London, UK), and VISCOM SARL (Saint Ouen l'Aumône, France). Threshold values between ossified parts and soft tissues were substantial and easily allowed osteological reconstructions. Three-dimensional (3D) rendering and visualization were performed using Drishti v.1.0 (Drishti Paint and Render, Limaye 2006). All the results obtained from 3D reconstructions were checked through the acquisition of shadow images, comparable to a conventional high-resolution X-ray as described in Weisbecker et al. (2008). Ossification centers were readily apparent in both 3D reconstructions and shadow X-rays.

Quantification of developmental trajectories

The sequence of ossification of a number of specific elements is given in Tables 2 and 3. We used two methods to quantify sequence heterochrony: sequence analysis of variance (ANOVA) and event pairing/Parsimov.

Sequence-ANOVA was used by Nunn and Smith (1998), Smith (2001), and Keyte and Smith (2010), and requires that every species be sampled for the same series of elements. The first step consists in constructing the developmental sequence by ordering the events by their relative stage for each taxon. This method uses standard nonparametric ranking procedures to deal with tied ossification events. In the case of ties, we used the average rank for the tied events (Siegel and Castellan 1988). For instance, if three ossification events occur simultaneously at fourth in the overall series, each would receive a rank of 5 (i.e., $[4 + 5 + 6]/3$). If in the same series the next two characters occur simultaneously at fifth place, their rank would be 7.5 (i.e., $[7 + 8]/2$). The dataset is then converted into transformed ranks (presented in Tables 4 and 5 for our samples). The ranked dataset is then plotted graphically, illustrating the major differences across species and enabling statistical scrutiny. Smith (2001) used ANOVA to recognize characters that show significantly more differences in rank position between than within groups (see also Nunn and Smith 1998). We checked the results given by ANOVA by running the same analysis with a nonparametric Mann–Whitney *U*-test and recovered the same results. These methods provide a quantitative approach to detect events that are advanced or delayed in one group relative to another. Sequence-ANOVA allows only the determination of the existence of a heterochronic shift. It conveys information on the direction of a shift not in absolute terms, but compared with an explicit reference taxon. Shifts identified by sequence-ANOVA are therefore discussed here in terms of "earlier" and "later"

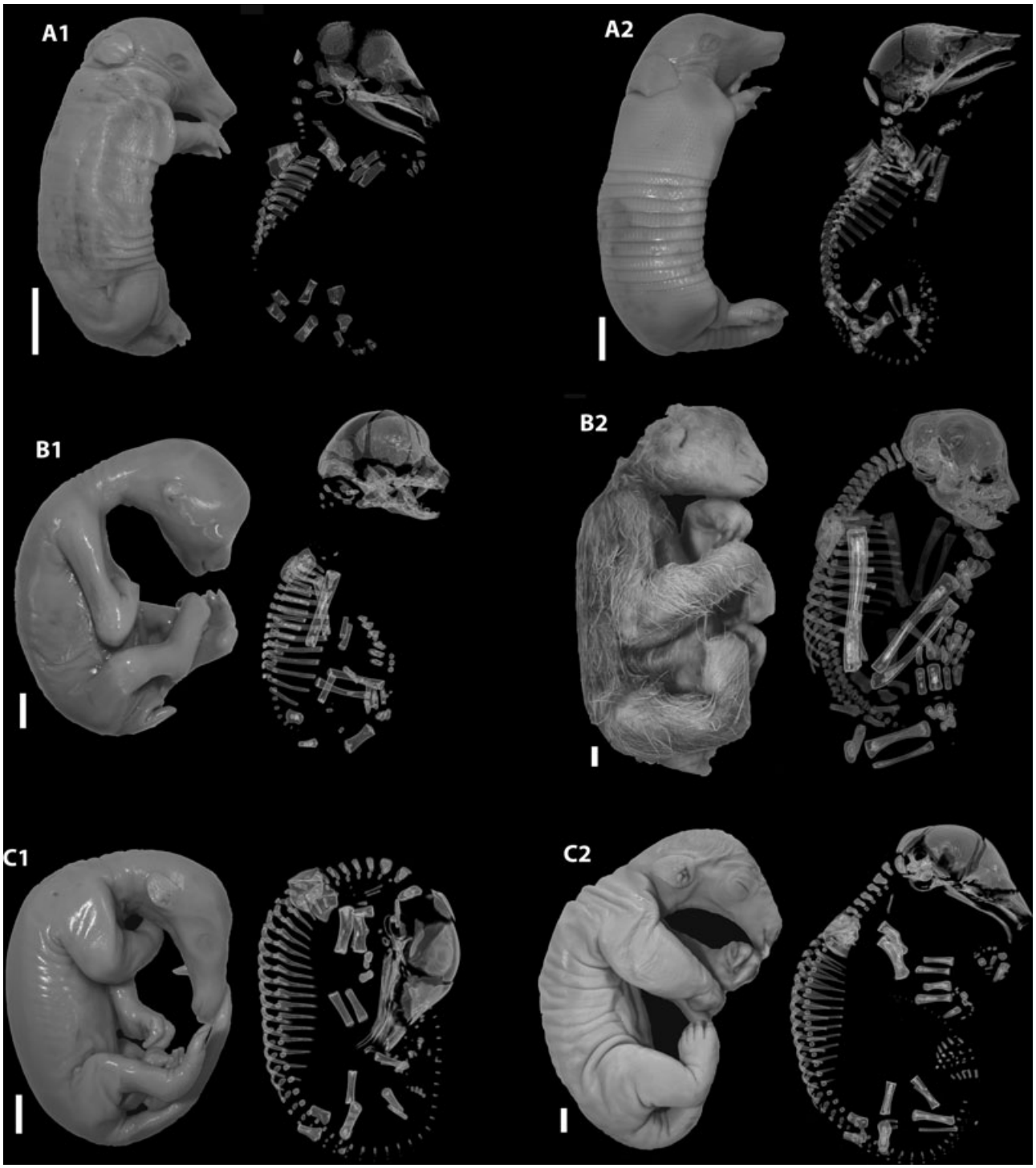

Fig. 1. Representative ontogenetic stages of xenathrans. Lateral view of specimens (left) and three-dimensional reconstruction of CT-scans of skeleton (right) in (A) nine-banded armadillos *Dasypus novemcinctus*: A1, ZMB 85893 CRL = 43 mm and A2, ZMB A5022 CRL = 74 mm; (B) *Bradypus variegatus*: B1, ZMB 41122 CRL = 70 mm and B2, ZMB 41120 CRL = 170 mm; (C) *Tamandua tetradactyla*: C1, ZMB A5023 CRL = 95 mm and C2, ZMB 40639 CRL = 125 mm. Scale bars = 1 cm.

Table 1. Sources of data used in the analysis of ossification sequence with specimen and stage numbers

Species name	Specimen numbers/stages		References
	Cranial	Postcranial	
Sauropsida			
Alligator mississippiensis	36/7	*Al/1*	Rieppel (1993a)
Lacerta vivpara	23/6	36/9	Rieppel (1993b)
Coturnix coturnix	15/4	-	Nakane and Tsudzuki (1999)
Xenarthra			
Bradypus variegatus	4/2	4/4	Present study; Schneider (1955)
Choloepus didactylus	5/1	5/4	Present study
Cyclopes didactylus	5/2	5/5	Present study
Tamandua tetardactyla	4/4	4/4	Present study
Dasypus novemcinctus	32/8	32/14	Present study
Euarchontoglires			
Tupaia javanica	24/6	-	Zeller (1987), Nunn and Smith (1998), Goswami (2007)
Tarsius spectrum	21/6	-	Nunn and Smith (1998)
Homo sapiens	60/	60/17	Mall (1906), Davies and Parsons (1927)
Rattus norvegicus	N.a./6	N.a./14	Strong (1925)
Mus musculus	N.a./7	41/5	Johnson (1933), Theiler (1972), Patton and Kaufman (1995), Kaufman (2008)
Cavia porcellus	N.a./12	N.a./12	Petri (1935), Wilson et al. (2010)
Mesocricetus auratus	168/7	168/8	Beyerlein et al. (1951), Kanazawa and Mochizuki (1974)
Meriones unguiculatus	9/5	187/8	Yukawa et al. (1999), Sánchez-Villagra et al. (2008)
Peromyscus melanophrys	13/5	7/4	Sánchez-Villagra et al. (2008), Weisbecker et al. (2008)
Octodon degus	-	8/5	Wilson et al. (2010)
Rhabdomys pumilio	61/12	61/11	Wilson et al. (2010)
Laurasiatheria			
Myotis lucifugus	-	19/7	Adams (1992)
Rousettus amplexicaudatus	11/7	12/10	Sánchez-Villagra et al. (2008), Weisbecker et al. (2008)
Cryptotis parva	15/11	-	Sánchez-Villagra et al. 2008
Bos taurus	-	180/9	Lindsay (1969a, 1969b)
Sus scrofa	10/7	N.a./12	Stöckli (1922), Nunn and Smith (1998)
Talpa europaea	16/7	22/9	Prochel (2006), Goswami and Prochel (2007), Prochel et al. (2008), Sánchez-Villagra et al. (2008)
Felis domestica	17/7	-	Nunn and Smith (1998)
Manis javanica	12/4	-	Nunn and Smith (1998)
Marsupiala			
Didelphis virginiana	16/6	16/9	de Oliveira et al. (1998)
Trichosurus vulpecula	6/4	32/9	Weisbecker et al. (2008)
Macropus eugenii	20/6	11/9	Nunn and Smith (1998), Weisbecker et al. (2008)
Dasyurus viverrinus	18/7	19/10	Nunn and Smith (1998), Goswami (2007), Weisbecker et al. (2008)
Sminthopsis macroura	-	11/8	Frigo and Wooley (1996)
Antechinus stuartii	-	22/10	Weisbecker et al. (2008)
Cercartetus concinnus	-	25/8	Weisbecker et al. (2008)
Isoodon macrourus	-	15/10	Weisbecker et al. (2008)
Petaurus breviceps	-	22/6	Weisbecker et al. (2008)
Vombatus ursinus	-	9/6	Weisbecker et al. (2008)
Caluromys philander	9/6	-	Goswami (2007), Sánchez-Villagra et al. (2008)
Perameles nasuta	10/9	-	Nunn and Smith (1998), Goswami (2007)
Monodelphis domestica	28/8	-	Nunn and Smith (1998), Goswami (2007)

relative to this reference taxon. In the present study, sequence-ANOVA illuminates the pattern of change of different cranial and postcranial elements of xenarthrans relative to the developmental trajectory of other placental and marsupial mammals (Figs. 3 and 4). However, the ranking procedure used has disadvantages. When ties accumulate within the ontogeny of any single taxon (exacerbated in species sampled by relatively few ontogenetic stages), it will tend to increase considerably the value of the transformed rank and to create artifactual heterochronies. In our sample, this occurs primarily due to lack of coverage of the

Table 2. Relative timing of onset of ossification (ranks) in the cranial elements for all species examined and compiled from the literature

	Bradypus	Choloepus	Dasypus	Cyclopes	Tamandua	Tupaia	Tarsius	Homo	Rattus	Mus	Peromyscus	Meriones	Mesocricetus	Rhabdomys	Cavia	Talpa	Cryptotis	Rousetus	Felis	Sus	Manis	Monodelphis	Caluromys	Didelphis	Parameles	Dasyurus	Macropus	Trichosurus	Alligator	Lacerta	Coturnix
Premaxilla	1	1	1	1	1	1	1	2	2	1	1	1	2	2	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	2
Maxilla	1	1	1	1	1	1	1	1	1	1	1	2	1	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2
Palatine	2	1	1	1	1	4	2	4	2	1	2	3	2	1	1	1	2	3	?	?	?	3	3	2	3	3	?	3	3	1	2
Dentary	1	1	1	1	1	1	?	1	1	1	1	1	1	1	1	1	1	1	?	?	?	1	1	2	?	?	?	?	1	1	1
Frontal	1	1	1	1	1	2	2	3	1	1	2	1	2	1	1	1	2	2	2	2	1	2	2	3	3	2	2	2	2	2	2
Parietal	1	1	1	1	1	2	3	3	2	1	2	4	2	1	1	1	3	2	3	3	2	4	2	3	5	4	3	4	5	2	3
Squamosal	1	1	1	1	1	3	2	3	2	3	4	4	2	6	1	1	5	1	4	1	2	3	3	6	4	4	2	2	2	2	1
Basioccipital	2	1	3	1	1	4	6	6	2	1	2	2	3	3	2	1	6	4	5	5	3	4	4	3	6	5	4	4	6	4	3
Nasal	1	1	1	1	1	4	?	4	3	3	4	3	3	5	2	1	4	3	?	?	?	4	3	5	4	5	?	3	4	2	1
Pterygoid	1	1	2	?	1	5	?	4	2	1	2	2	?	1	3	1	3	4	?	?	?	7	5	3	5	5	?	3	1	1	1
Exoccipital	2	1	5	1	1	3	4	3	2	1	3	3	2	4	3	2	6	4	5	3	3	2	3	3	3	3	2	3	3	3	3
Basisphenoid	4	1	7	?	2	6	6	8	4	3	3	4	2	7	4	2	7	5	6	6	4	6	4	3	8	6	5	4	3	4	3
Jugal	1	1	1	?	1	2	2	3	3	2	4	4	5	8	1	3	11	2	3	4	1	3	2	3	2	3	2	2	1	2	1
Lacrimal	2	1	1	1	1	4	3	7	3	4	4	4	6	9	3	4	6	3	?	?	?	3	4	4	6	5	?	3	2	5	?
Alisphenoid	3	1	4	2	1	4	5	5	5	1	4	4	4	10	1	6	8	5	5	3	3	5	3	?	5	4	3	3	?	3	?
Orbitosphenoid	4	1	6	2	3	5	?	7	6	5	5	5	7	11	5	7	10	7	?	?	?	7	5	?	7	6	?	4	7	6	4
Periotic	4	1	8	2	4	7	7	4	?	6	5	?	?	12	5	5	9	6	7	7	4	8	6	?	9	7	6	4	6	4	4

Table 3. Relative timing of onset of ossification (ranks) in the postcranial elements for all species examined and compiled from the literature

	Bradypus	Choloepus	Dasypus	Cyclopes	Tamandua	Homo	Rattus	Mus	Peromyscus	Meriones	Mesocricetus	Rhabdomys	Cavia	Octodon	Talpa	Cryptotis	Myotis	Rousetus	Sus	Bos	Monodelphis	Didelphis	Dasyurus	Macropus	Trichosurus	Wombatus	Antechinus	Isoodon	Petaurus	Cercartetus	Sminthopsis	Alligator	Lacerta
Clavicle	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	?	1	1	1	1	1	2	1	1	1	1	1	1	?	1
Humerus	1	1	1	1	1	2	2	2	2	2	2	1	2	1	3	3	2	1	1	2	2	2	1	1	2	1	1	1	1	1	1	1	1
Ribs	1	1	1	1	1	5	2	2	?	3	3	1	3	1	1	2	3	1	3	3/4	2	2	1	1	2	1	1	1	1	1	2	4	3
Femur	1	1	1	1	1	2	3	2	2	2	3	1	2	1	3	3	2	1	1	2	5	5	2	2	3	2	2	3	1	2	3	1	1
Radius	1	1	3	1	1	3	2	2	2	2	3	1	2	1	2	3	2	1	1	3	2	2	1	1	2	1	1	1	1	1	1	1	1
Ulna	1	1	1	1	1	1	2	2	2	2	3	1	2	1	2	3	2	1	1	3	2	2	1	1	2	1	1	1	1	1	1	1	1
Scapula	1	1	1	1	1	5	3	2	2	2	3	1	3	1	2	3	3	1	2	3	2	2	1	1	2	1	1	1	1	1	1	4	3
Cervic.	1	1	6	1	1	7	3	2	2	2	3	1	4	1	2	3	3	3	4	4	2	2	1	1	2	1	1	1	1	1	2	3	3
Thorac.	1	1	7	1	2	7	3	2	2	3	3	2	5	2	2	3	3	5	4	4	3	2	1	1	2	1	1	1	1	1	2	4	5
Tibia	1	1	2	1	1	3	3	T	2	2	3	1	2	1	2	3	2	1	2	3	5	5	2	2	3	2	2	2	1	2	3	1	1
Fibula	1	1	2	1	1	5	3	2	2	2	3	2	2	1	2	3	2	2	2	3	5	5	2	2	3	2	2	2	1	2	3	1	1
Lumbar	1	1	8	1	2	9	4	2	2	3	3	3	5	2	4	4	3	5	4	3/4	4	3	2	2	3	2	2	2	1	2	3	4	5
Sacral	1	1	10	1	2	11	4	3	2	4	4	5	8	3	6	4	6	7	4	3/4	5	5	2	4	4	2	3	2	1	2	3	5	5
Caudal	3	2	10	1	2	14	4	3	3	6	6	10	10	3	7	5	6	7	6	5	6	5	3	4	4	3	3	3	2	3	4	5	6
Ilium	1	1	4	1	1	6	3	2	2	3	3	2	4	1	5	4	3	5	2	4	5	5	2	4	2	2	2	2	1	2	3	6	3
Man. Phal.	1	1	1	2	2	6	7	3	3	5	4	9	6	2	8	4	5	6	5	4	2	5	3	1	1	3	2	2	2	2	3	7	3
Ped. Phal.	1	1	5	2	2	8	7	4	3	5	4	10	7	3	8	6	3	6	7	4	8	6	5	3	3	2	7	2	2	2	5	7	2
Ischium	3	1	10	2	2	12	5	3	3	5	6	4	5	1	8	4	5	7	8	4	7	6	4	2	4	3	3	4	2	4	3	6	7
Pubis	2	1	10	2	2	15	5	3	3	8	6	7	10	4	8	6	5	6	11	8	8	7	8	7	8	5	8	7	3	7	6	7	4
Metac	4	1	9	2	2	7	4	3	3	4	4	6	6	3	8	6	3	4	5	4	6	4	3	3	4	2	4	4	1	4	3	5	3
Metat	3	1	10	2	2	8	4	3	3	5	6	7	7	3	8	7	4	7	7	4	8	6	5	6	6	4	6	5	3	5	4	2	2
Tarsals	3	3	12	3	3	10	6	4	4	7	7	10	11	4	8	7	6	8	9	7	9	8	9	8	7	4	9	6	4	6	7	9	6
Carpals	4	4	13	5	4	16	8	5	4	8	8	11	12	5	9	7	7	10	12	9	10	8	10	9	9	6	10	7	5	7	8	8	8
Sternum	4	4	11	4	4	13	5	3	3	?	5	8	9	3	6	4	3	9	10	6	8	6	6	6	6	4	5	5	3	5	5	10	9
Epipubics	5	5	14	6	5	17	9	6	5	9	9	12	13	6	10	8	8	11	13	10	5	9	4	5	5	4	6	8	6	8	4	10	9

Table 4. Ranked data for timing of cranial events

	Bradypus	Dasypus	Tamandua	Tupaia	Homo	Mus	Peromyscus	Rhabdomys	Cavia	Talpa	Cryptotis	Rousetus	Monodelphis	Caluromys
Premaxilla	5	5.5	7.5	2	3	5.5	2	6.5	5	5.5	2	8.5	2	2
Maxilla	5	5.5	7.5	2	1.5	5.5	2	6.5	5	5.5	2	4.5	2	2
Palatine	11.5	5.5	7.5	11	10.5	5.5	6	3	5	5.5	4.5	8.5	7.5	9
Dentary	5	5.5	7.5	2	1.5	5.5	2	3	5	5.5	2	1.5	2	2
Frontal	5	5.5	7.5	5	6	5.5	6	3	5	5.5	4.5	4.5	4.5	5
Parietal	5	5.5	7.5	5	6	5.5	6	3	5	5.5	6.5	4.5	11	5
Squamosal	5	5.5	7.5	7.5	6	13	13	11	5	5.5	9	1.5	7.5	9
Basioccipital	11.5	12	7.5	11	14	5.5	6	8	10.5	5.5	11	12	11	13
Nasal	5	5.5	7.5	11	10.5	13	13	10	10.5	5.5	8	8.5	11	9
Pterygoid	5	11	7.5	14.5	10.5	5.5	6	3	13	5.5	6.5	12	15.5	15.5
Exoccipital	11.5	14	7.5	7.5	6	5.5	9.5	9	13	11.5	11	12	4.5	9
Basisphenoid	16	16	15	16	17	13	9.5	12	15	11.5	13	14.5	14	13
Jugal	5	5.5	7.5	5	6	11	13	13	5	13	17	4.5	7.5	5
Lacrimal	11.5	5.5	7.5	11	15.5	15	13	14	13	14	11	8.5	7.5	13
Alisphenoid	14	13	7.5	11	13	5.5	13	15	5	16	14	14.5	13	9
Orbitosphenoid	16	15	16	14.5	15.5	16	16.5	16	16.5	17	16	17	15.5	15.5
Periotic	16	17	17	17	10.5	17	16.5	17	16.5	15	15	16	17	17

earliest ossification events, that is, leading to a number of events tied at #1. We took into account the potential for such artifactual "significance" of heterochronies resulting from the accumulation of events tied at the beginning of a given ontogenetic series.

Event-pair analyses were performed in the phylogenetic context shown in Fig. 2. Following previous studies (e.g. Sánchez-Villagra et al. 2007; Weisbecker et al. 2008), we constructed two separate data matrices for the postcranial and cranial datasets. For all species, an event-pair matrix was produced based on the ossification sequences in which the ossification onset in the 17 cranial elements and 25 postcranial elements was compared with every other event. Two separate data matrices were obtained: one with $^1/_2$ $(17^2 - 17) = 136$ events for cranial elements and the other with $^1/_2$ $(25^2 - 25) = 300$ event pairs for the postcranial elements. Three character states were used to represent the relative timing of one event relative to another: 0, 1, and 2, corresponding to prior, simultaneous, or subsequent ossification of one element relative to another (respectively). We used Parsimov (Jeffery et al. 2005) in order to document the patterns of change in event pairs. This program employs a parsimony approach to search for the minimal amount of heterochrony required to explain sequence differences between species. (Jeffery et al. 2005). Due to the search for minimum heterochrony, Parsimov is conservative. However, it has a track record of providing a robust estimate of heterochronic change, even when the dataset includes missing data or many ties (Sánchez-Villagra 2002; Sánchez-Villagra et al. 2008; Weisbecker et al. 2008; Weisbecker and Mitgutsch 2010). The considerable number of ties in the event-pair dataset required analysis with and without ties (see below). We therefore did not use the newer Pgi heterochrony search algorithm by Harrison and Larsson (2008), which is currently not programmed to analyze datasets with ties excluded (Harrison, personal communication).

We ran the analyses using both ACCTRAN and DELTRAN optimizations as recommended by Jeffery et al. (2005). The ACCTRAN option assumes accelerated transformations (favoring reversals); the DELTRAN option provides delayed transformations (favoring convergences; Maddison and Maddison 1992). Only the events that were reported using both approaches were interpreted as heterochronies, although we also examined the extent to which results from one or the other reflected results from sequence-ANOVA. The consensus results of ACCTRAN and DELTRAN event shifts in the onset of ossification of cranial and postcranial elements are presented in the supporting information (Figs. S2–S5). As for the sequence-ANOVA method, the accumulation of ties increases the probability of artifactual "significance" of heterochronic shifts that are not directly observable. In order to take into account this methodological artifact, we decided to run two Parsimov analyses, one with the original data and a second with all ties converted from score "1" to score "?" (i.e., unknown timing—Sánchez-Villagra et al. 2009). The results are presented in supporting information (Figs. S2–S5).

Because these procedures require a fairly dense series of developmental stages, the silky anteater *Cyclopes* and the two-toed sloth *Choloepus* were considered only for analyses involving the postcranial skeleton. Using the results of Schneider (1955), we included the three-toed sloth (*Bradypus*) in the analyses considering cranial elements.

Table 5. Ranked data for timing of postcranial events

	Bradypus	Choloepus	Dasypus	Cyclopes	Tamandua	Homo	Rattus	Mus	Peromyscus	Mesocricetus	Rhabdomys	Cavia	Octodon	Talpa	Cryptotis	Myotis	Rousetus	Bos	Monodelphis	Didelphis	Dasyurus	Macropus	Trichosurus	Wombatus	Antechinus	Isoodon	Petaurus	Cercartetus	Sminthopsis
Clavicle	8.5	10.5	4	8	6	1.5	1	1	1	1	5	1	6.5	1.5	1	1	4	1	1	1	4.5	5	5.5	5	4.5	4.5	8	4.5	3
Humerus	8.5	10.5	4	8	6	3.5	3.5	7.5	8	2.5	5	4.5	6.5	10.5	7	4.5	4	2.5	5	5	4.5	5	5.5	5	4.5	4.5	8	4.5	3
Ribs	8.5	10.5	4	8	6	8	3.5	7.5	8	2.5	5	8.5	6.5	1.5	2	12	4	7.5	5	5	4.5	5	5.5	5	4.5	4.5	8	4.5	7
Femur	8.5	10.5	4	8	6	3.5	9	7.5	8	8.5	5	4.5	6.5	10.5	7	4.5	4	2.5	13	14	11.5	12.5	12	13.5	11	16.5	8	12.5	13
Radius	8.5	10.5	10	8	6	5.5	3.5	7.5	8	8.5	5	4.5	6.5	6	7	4.5	4	7.5	5	5	4.5	5	5.5	5	4.5	4.5	8	4.5	3
Ulna	8.5	10.5	4	8	6	1.5	3.5	7.5	8	8.5	5	4.5	6.5	6	7	4.5	4	7.5	5	5	4.5	5	5.5	5	4.5	4.5	8	4.5	3
Scapula	8.5	10.5	4	8	6	8	9	7.5	8	8.5	5	8.5	6.5	6	7	12	4	7.5	5	5	4.5	5	5.5	5	4.5	4.5	8	4.5	3
Cervic.	8.5	10.5	13	8	6	13	9	7.5	8	8.5	5	10.5	6.5	6	7	12	10	15.5	5	5	4.5	5	5.5	5	4.5	4.5	8	4.5	7
Thorac.	8.5	10.5	14	8	16.5	13	9	7.5	8	8.5	11	13	14	6	7	12	13	15.5	9	5	4.5	5	5.5	5	4.5	4.5	8	4.5	7
Tibia	8.5	10.5	8.5	8	6	5.5	9	7.5	8	8.5	5	4.5	6.5	6	7	4.5	8.5	7.5	13	14	11.5	12.5	12	13.5	11	12	8	12.5	13
Fibula	8.5	10.5	8.5	8	6	8	9	7.5	8	8.5	11	4.5	6.5	6	7	4.5	8.5	7.5	13	14	11.5	12.5	12	13.5	11	12	8	12.5	13
Lumbar	8.5	10.5	15	8	16.5	17	15	7.5	8	8.5	13	13	14	12	14.5	12	13	7.5	10	9	11.5	12.5	12	13.5	11	12	8	12.5	13
Sacral	8.5	10.5	19	8	16.5	19	15	17.5	8	15.5	15	19	18.5	14.5	14.5	22	19.5	7.5	13	14	11.5	18.5	17	13.5	15.5	12	8	12.5	13
Caudal	19.5	21	19	8	16.5	22	15	17.5	18.5	20.5	22	21.5	18.5	16	18	22	19.5	20	16.5	14	16	18.5	17	18.5	15.5	16.5	17.5	17	18.5
Ilium	8.5	10.5	11	8	6	10.5	9	7.5	8	8.5	11	10.5	6.5	13	14.5	12	13	15.5	13	14	11.5	12.5	17	13.5	11	12	8	12.5	13
Man. Phal.	8.5	10.5	4	18.5	16.5	10.5	22.5	17.5	18.5	15.5	20	15.5	14	20	14.5	19	16	15.5	5	14	16	5	1	5	15.5	12	17.5	12.5	13
Ped. Phal.	8.5	10.5	12	18.5	16.5	15.5	22.5	22.5	18.5	15.5	22	17.5	18.5	20	20	12	16	15.5	20.5	19.5	19.5	16.5	12	13.5	21	12	17.5	12.5	20.5
Ischium	19.5	10.5	19	18.5	16.5	20	19	17.5	18.5	20.5	14	13	6.5	20	14.5	19	19.5	15.5	18	19.5	18	12.5	17	18.5	15.5	18.5	17.5	18.5	13
Pubis	17	10.5	19	18.5	16.5	23	19	17.5	18.5	20.5	17.5	21.5	22.5	20	20	19	16	23	20.5	22	22	22	23	23	22	23.5	21	23.5	22
Metac	23	10.5	16	18.5	16.5	13	15	17.5	18.5	15.5	16	15.5	18.5	20	20	12	11	15.5	16.5	10	16	16.5	17	13.5	18	18.5	8	18.5	13
Metat	19.5	10.5	19	18.5	16.5	15.5	15	17.5	18.5	20.5	17.5	17.5	18.5	20	23	17	19.5	15.5	20.5	19.5	19.5	20.5	20.5	21	20	20.5	21	20.5	18.5
Tarsals	19.5	22	23	22	22	18	21	22.5	23.5	23	22	23	22.5	20	23	22	22	22	23	23.5	23	23	22	21	23	22	23	22	23
Carpals	23	23.5	24	24	23.5	24	24	24	23.5	24	24	24	24	24	23	24	24	24	24	23.5	24	24	24	24	24	23.5	24	23.5	24
Sternum	23	23.5	22	23	23.5	21	19	17.5	18.5	18	19	20	18.5	14.5	14.5	12	23	21	20.5	19.5	21	20.5	20.5	21	19	20.5	21	20.5	20.5

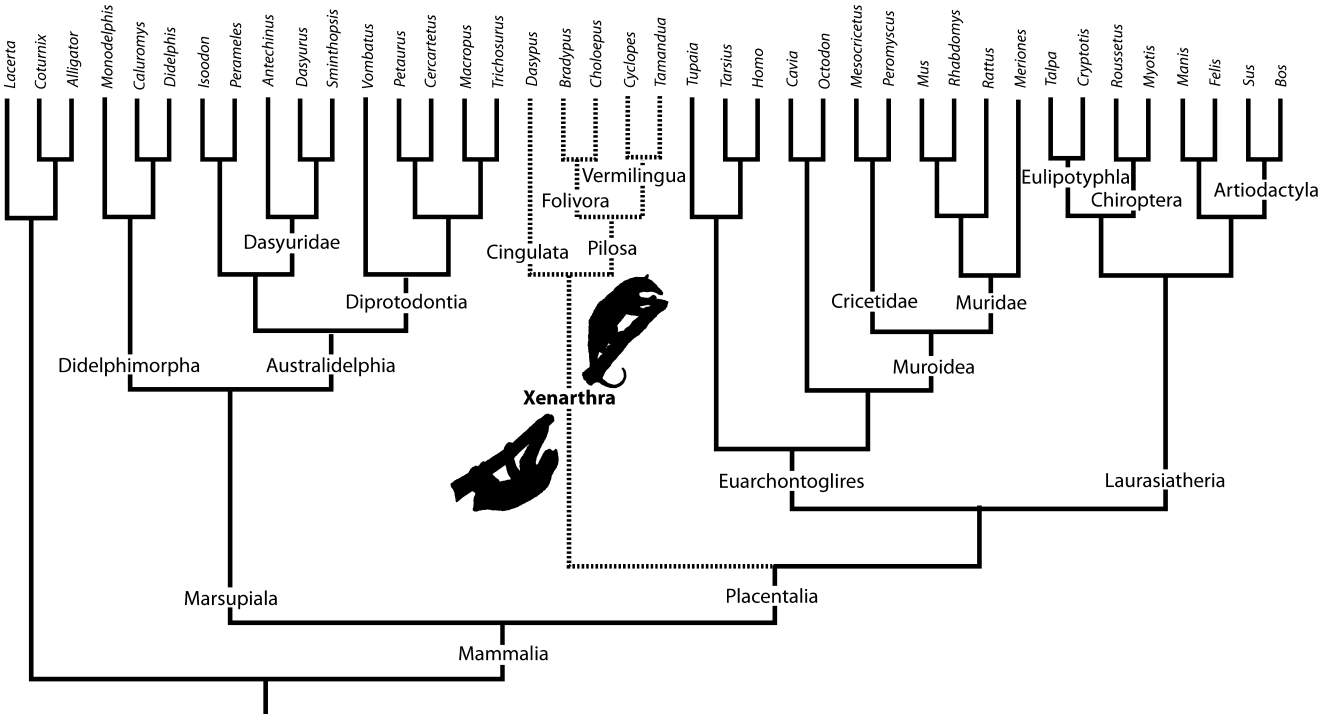

Fig. 2. Phylogenetic relationships among the species studied in this analysis, phylogeny reconstructed from morphological and molecular analyses (Phillips et al. 2006; Möller-Krull et al. 2007; Prasad et al. 2008). Species not included in this analysis (e.g., Afrotheria) are not included on the tree.

RESULTS

Skeletogenesis in Dasypus

Cranially, *D. novemcinctus* displays a similar ossification sequence as that of other placental mammals. Our growth series exhibits a concentration of ossification events within the first few stages with 10 of 17 elements (70%) ossifying first, followed by the pterygoid and then the basioccipital. Bones of the rostrum ossify early, before those of the basicranium and posterior skull (Table 2). The alisphenoid ossifies after the basioccipital and is followed by the exoccipital and the orbistosphenoid. The basicranium is the last region to start its ossification, the basisphenoid being the penultimate bone to ossify, followed by the periotic, similar to the pattern observed in most other mammals considered here.

In our series of *D. novemcinctus*, 7 of 25 elements (40%) of the postcranial skeleton ossify first (rank 1, Table 3). A similar concentration of relative simultaneity for the earliest events was also found in other mammals (Weisbecker et al. 2008; Wilson et al. 2010). Specifically, the initial ossifications reported here involve the clavicle, humerus, ribs, femur, ulna, scapula, and manual phalanges. Following these, the tibia and the fibula ossify second, followed by the radius and ilium. The pedal phalanges ossify fifth, followed by elements of the spine (cervical, thoracic, then lumbar vertebrae), and the metacarpals. Then, the ischium, pubis, metatarsals, and sacral and caudal vertebrae start their ossification simultaneously. The ossification of the sternum is next, followed by the tarsals, and then the carpals. This pattern is widespread among placentals (Weisbecker et al. 2008; Wilson et al. 2010).

Heterochrony in Xenarthra as determined by sequence-ANOVA

Only two marsupial sequences (*Monodelphis* and *Caluromys*) display ranks for all cranial elements. In addition, ANOVA requires more than two sequences to be known (Nunn and Smith 1998). For these reasons, we cannot meaningfully compare these results to the data on cranial ossification in marsupials as a whole using sequence ANOVA. Relative to other placentals, onset of ossification in 3 of the 17 cranial elements differs statistically in xenarthrans (based on data from *Dasypus*, *Bradypus*, and *Tamandua*—Fig. 3). Specifically, we find a late ossification of the dentary and early ossifications of the nasal and lacrimal. The late ossification of the dentary could be explained by the concentration of ties in the first rank due to an artifact of sampling in earliest stages. The dentary is characterized by an early ossification in all mammals and was previously recognized as one of the least variable cranial bones (Sánchez-Villagra et al. 2008). Given

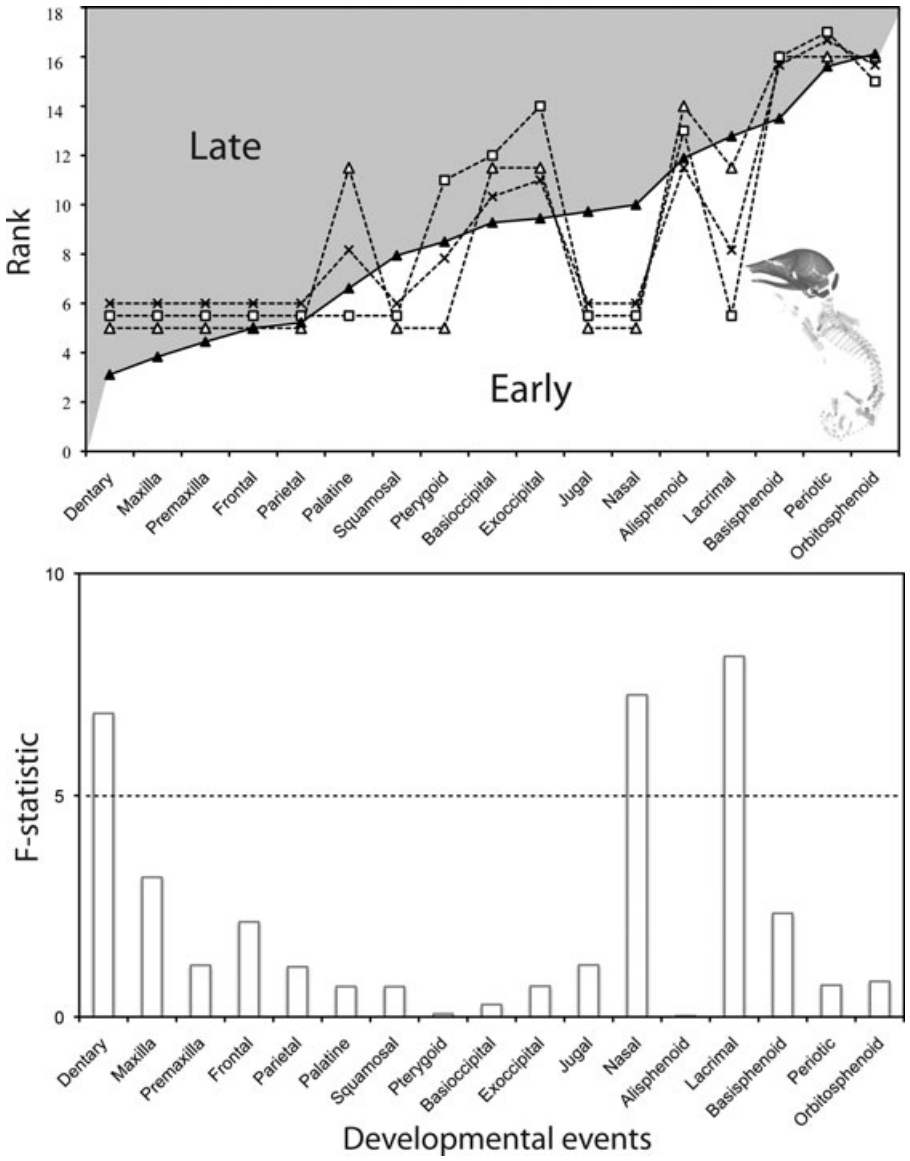

Fig. 3. Ossification sequence of cranial elements in xenarthrans relative to the mean rank of placentals (solid triangles). Above, the mean rank of *Bradypus*, *Dasypus*, and *Tamandua* are represented by crosses. The ossification sequences of *Dasypus* (open squares) and *Bradypus* (open triangles) were added for comparisons. Results of the ANOVAs between xenarthrans (crosses) and other placentals (solid triangles) with *F*-statistics are shown below. The dotted line represents $P < 0.05$. Shifts can be observed between the two groups, and are statistically significant when they exceed the dotted line (early ossification of lacrimal and nasal, late ossification of dentary). The dotted line gives the statistical calculation of $P < 0.05$. Shifts can be observed between the two groups without being statistically significant (e.g. exoccipital, jugal, basisphenoid). Because statistical analyses that consider variance between two groups (ANOVA) cannot be used to compare the ossification of a single taxon, the study of the sequence heterochrony does not convey significance values for the genera *Dasypus* and *Bradypus*. (Significance of events tied at rank #1 is potentially artifactual; see text.)

that the nasal and lacrimal also ossified first in both *Dasypus* and *Tamandua*, the significance of their early ossification could also be considered as unresolvable without further data. The low resolution of our cranial ossification sequences in xenarthrans did not allow the detection of any unambiguous heterochronic shifts for cranial elements between xenarthrans and other mammals. The ties occurred exclusively in the earliest stages, and will hopefully be resolved with future studies using more developmental stages.

ANOVA can only be used to compare the ossification between two groups. Thus, we compared statistically the differences between all xenarthrans and all other placentals (Fig. 3, lower part), but plotted the developmental trajectory of *Bradypus* and *Dasypus*, which displayed well-resolved developmental sequences in order to compare their trajectory to that of Xenarthra as a whole (Figs. 3 and 4). The sequence analysis for their cranial elements reveals a late ossification of the palatine, exoccipital, alisphenoid, basioccipital, and basisphenoid, as well as an early ossification of the squamosal, pterygoid, and jugal in both genera.

The postcranial sequences are much more resolved than those for the cranium. Specifically, 5 of 24 postcranial elements differ statistically in xenarthrans and other placentals (Fig. 4, A and B, lower part), whereas we find 15 significant differences between xenarthrans and marsupials. Xenarthrans differ from other placentals by a late ossification of the sternum and clavicle, and an early ossification of pubis, pedal, and manual phalanges (Fig 4A); they differ from marsupials by a late ossification of the clavicle, humerus, radius, ulna, scapula, ribs, sternum, cervical, and thoracic

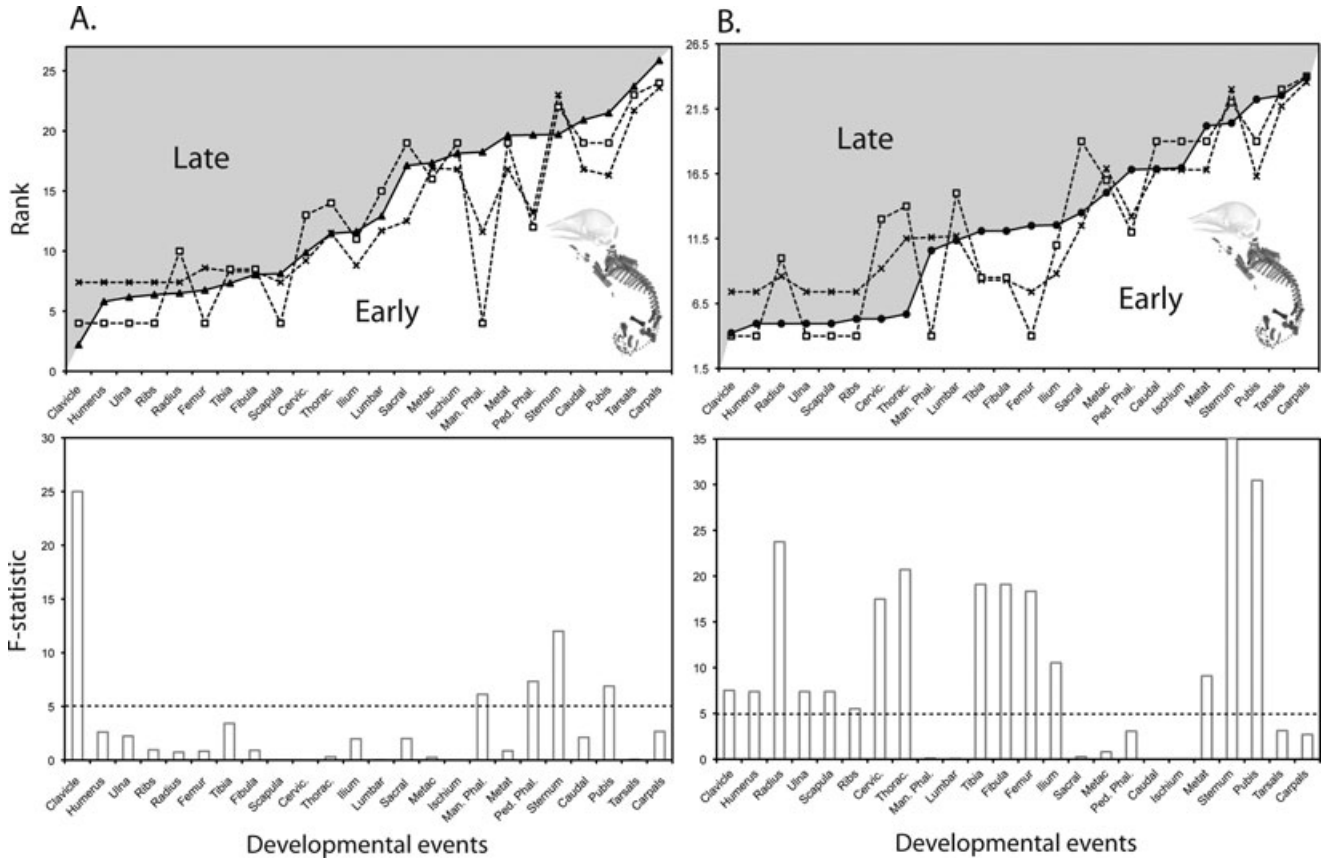

Fig. 4. Ossification sequence of postcranial elements in xenarthrans relative to the mean ranks of other placentals (A, solid triangles) and marsupials (B, solid circles). Above, mean ranks are represented by 5 xenarthrans (crosses), 13 placentals (solid triangles), and 11 marsupials (solid circles). The developmental trajectory of *Dasypus* (open squares) was added for comparison. Results of the ANOVAs between xenarthrans (crosses) and other placentals (A, solid triangles) or marsupials (B, solid circles), with *F*-statistics are shown below. The dotted line represents $P < 0.05$. Heterochronic shifts are statistically significant at $P < 0.05$ when they exceed the dotted line. Because statistical analyses that consider variance between two groups (ANOVA) cannot be used to compare the ossification of a single taxon, the study of the sequence heterochrony does not convey significance values for the genus *Dasypus*. (Significance of events tied at rank #1 is potentially artifactual; see text.)

vertebrae, and an early ossification of the tibia, fibula, femur, ilium, metatarsals, and pubis (Fig. 4B). As shown for the cranial elements, the shifts involving the clavicle, humerus, ulna, scapula, and ribs are most likely to be explained by the concentration of ties in the first fewer rank due to an artifact of sampling in the earliest stages. These elements ossify first across all sampled xenarthrans, but an increased density of ontogenetic stages would likely break up some or all of these ties.

The sequence analysis also reveals differences of *Dasypus* relative to both placentals and marsupials (Fig. 4) in comparison to differences observed for Xenarthra as a whole. Compared with other placentals, the nine-banded armadillo is characterized by a late ossification of radius, cervical, and thoracic vertebrae, and an early ossification of scapula, femur, pubis, and caudal vertebrae (Fig. 4A). It differs from marsupials by a late ossification of lumbar and sacral vertebrae, and an early ossification of pedal and manual phalanges (Fig. 4B).

Heterochrony in Xenarthra as determined by Parsimov

When ties are treated as missing data, Parsimov does not identify any heterochronic shift for cranial elements for the Xenarthra clade (Fig. S2), reflecting the results of sequence-ANOVA. However, when events coded as ties are included, *Dasypus* is characterized by an early heterochronic shift of the palatine with respect to frontal, parietal, and jugal, and an early ossification of the lacrimal in relation to frontal, parietal, squamosal, basioccipital, nasal, pterygoid, and jugal (Fig. S2). *Bradypus* is characterized by a late onset of ossification of the palatine with respect to premaxilla and nasal (Fig. S2). *Tamandua* is characterized by numerous au-

tapomorphic character shifts involving early onset of the parietal, basioccipital, exoccipital, lacrimal, and alisphenoid (Fig. S2). None of these shifts were retrieved with ties coded as missing data (Fig. S3).

For postcranial elements, only one early shift of the scapula in relation to the humerus occurs within Xenarthra (Fig. S4). This heterochronic shift occurs at a single developmental stage and should be considered as a methodological artifact that was detected by running the analyses without considering ties (Fig. S5). No heterochronic shifts were detected between sloths (Folivora) and anteaters (Vermilingua; see Figs. S4 and S5). With ties coded as present, *Dasypus* is characterized by an early ossification of the manual phalanges relative to the ilium, scapula, ribs, metacarpals, femur, ulna, and humerus, an early ossification of the pedal phalanges relative to metacarpals, and a late ossification of the radius compared to the fibula and tibia. *Bradypus* is characterized by a late ossification of the metacarpals relative to the ischium, metatarsals, sternum, and the caudal vertebrae. Interestingly, treating ties as missing data did not remove the significance of these events, and in fact showed the addition of a late ossification of the tibia relative to the scapula and ribs for *Dasypus* and an early ossification of the pubis relative to the ischium and metatarsals for *Bradypus*.

Choloepus is characterized by four heterochronic shifts that involve early ossifications of the pubis, ischium, metatarsals, and metacarpals (Fig. S4). Heterochronies that distinguish *Tamandua* include the late shift of the lumbar vertebrae compared to the metacarpals and the metatarsals, as well as the late shift of the thoracic vertebrae relative to the manual phalanges, the metatarsals, the metacarpals, and the cervical vertebrae. Several heterochronies distinguish *Cyclopes*, involving early ossifications of the thoracic, lumbar, sacral, and caudal vertebrae with respect to ribs and limb elements (Fig. S4). The significance of these shifts in *Choloepus*, *Tamandua*, and *Cyclopes* disappears when ties are treated as missing data.

Heterochrony in mammals

By comparing the developmental trajectory of the postcranial skeleton of xenarthrans with that of marsupials and other placentals (Fig. 4), we recover more significant differences between xenarthrans and marsupials than xenarthrans and other placentals. This confirms the well-known fact that substantial differences exist between the sequence of ossification of marsupials and placentals (Smith 2001; Sánchez-Villagra et al. 2008; Weisbecker et al. 2008). A comparison between placentals (excluding xenarthrans) and marsupials was then conducted to assign differences between both groups (Fig. 5). The results show that ossification of 12 of the 24 postcranial elements differs statistically in placentals and marsupials (Fig. 5). Only few of these significant shifts (clavicle and scapula) are due to ties occurring in the earliest stages. Compared to marsupials, placentals are characterized by a later ossification of the manual phalanges, metatarsals, sternum, pubis, cervical, thoracic, and caudal vertebrae, and display an early ossification of the tibia, fibula, and femur. The heterochronic shifts involving the tibia, fibula, and femur are highly significant ($P < 1 \times 10^{-5}$).

Otherwise, our Parsimov analysis identified few heterochronies among the major groups of mammals examined (Figs. S2–S5). No heterochronic shift is retrieved for the Theria clade. By retaining ties in the dataset, a late movement of the fibula, tibia, and femur with respect to the ilium, ulna, radius, and humerus, as well as a late movement of the metatarsals in relation to caudal vertebrae and ischium is reported for the Marsupialia clade, as reported in previous studies (Weisbecker et al. 2008). We recorded only one change in the onset of ossification of cranial elements among marsupials: the early ossification of the exoccipital in relation to the nasal. None of these heterochronic shifts were retrieved when ties were treated as missing data (Figs. S3–S5).

No heterochony was detected by Parsimov for the nodes representing Placentalia, Laurasiatheria, or Euarchontoglires. However, Parsimov does recover a shift of postcranial elements at the boreoeutherian node: the early ossification of the clavicle relative to the ulna and humerus (Fig. S4). Within Laurasiatheria, we discover two heterochronic shifts in both postcranial and cranial ossification sequence that characterize Artiodactyla: the early ossification of the squamosal in relation to the premaxilla and maxilla, and the late ossification of the fibula in relation to the femur and humerus. Once again, these heterochronic shifts were not retrieved by scoring ties as missing data (Fig. S5). As reported by Weisbecker et al. (2008), Lipotyphla is characterized by a large number of apomorphic sequence shifts that may relate to the stabilization of the anterior body axis in their relatively altricial neonates.

DISCUSSION

Event pairs and Parsimov

We found that Parsimov is sensitive to the treatment of event-pair ties (Figs. S2–S5). An abundance of "shifts" of the kind we observe in *Choloepus* and *Cyclopes* are features commonly observed in previous studies (Harrison and Larsson 2008; Wilson et al. 2010). A similar multiplication of shifts was previously reported for some rodent species (Wilson et al. 2010) and was interpreted as an artifact of crown nodes produced with the Parsimov algorithm (Harrison and Larsson 2008). This great number of autapomorphic shifts for both cranial and postcranial elements for the sloth-anteater clade (Pilosa; see Figs. S2-S5) could also result from limited sampling, as

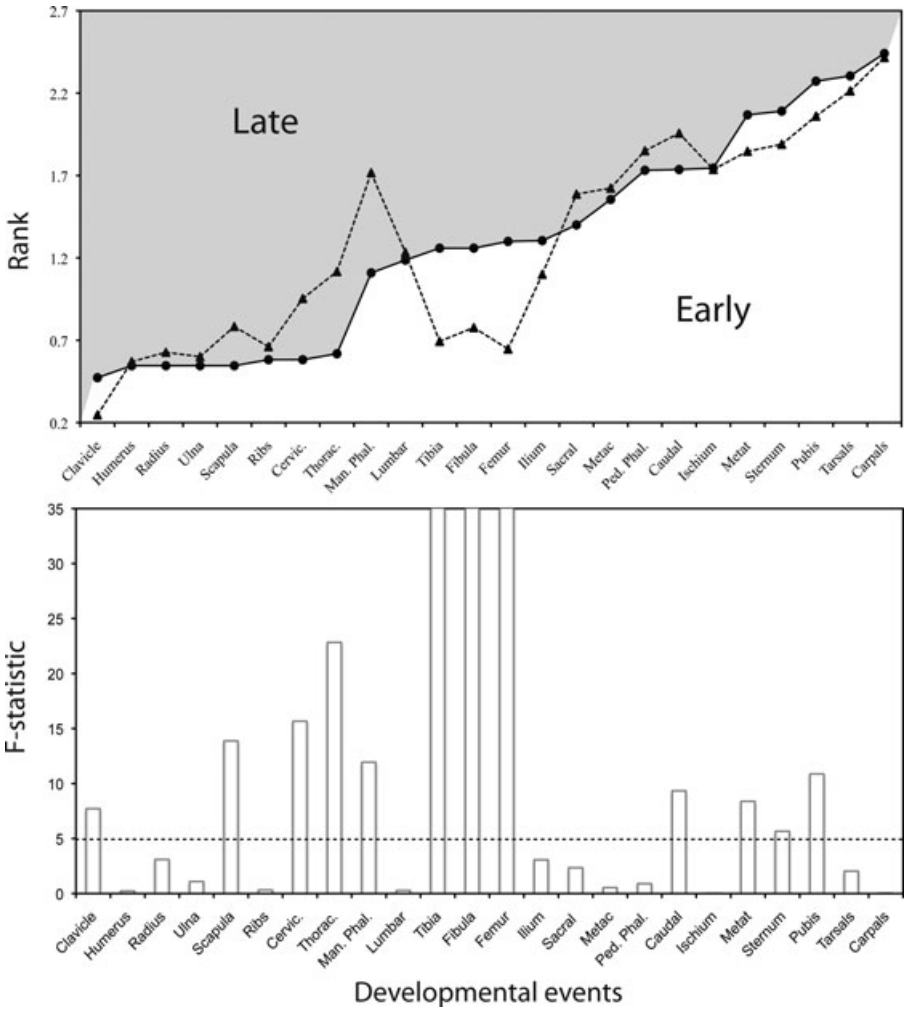

Fig. 5. Ossification sequence of postcranial elements in placentals (excluding xenarthrans, solid triangles) relative to the mean ranks of and marsupials (solid circles). Above, mean ranks are represented 13 placentals (solid triangles), and 11 marsupials (solid circles). Results of the ANOVAs with *F*-statistics are shown below. The dotted line represents $P < 0.05$. Heterochronic shifts are statistically significant at $P < 0.05$ when they exceed the dotted line. Xenarthran species were not included in this analysis in order to allow comparisons with previous studies (Sánchez-Villagra et al. 2009; Weisbecker et al. 2008).

they involve bones that ossify at the same rank. When ties are coded as missing, no such heterochronic shifts were detected (Figs. S3 and S5). While running Parsimov with missing data for ties successfully removes likely artifacts, it fails to retrieve demonstrable heterochronic shifts such as the relative timing of fore- and hind-limb ossification in marsupials (Weisbecker et al. 2008; Keyte and Smith 2010, and reference therein). It is possible that simultaneous events represent a real developmental pattern, particularly when heterochrony involving ties is detected in deeper nodes (Weisbecker et al. 2008). However, for the taxonomic scope and resolution in this study, it seems reasonable to conclude that Parsimov is overly conservative and suffers from a preponderance of type I errors (rejecting a valid null hypothesis), as noted in previous work (Harrison and Larsson 2008; Sánchez-Villagra et al. 2008; Weisbecker et al. 2008; Werneburg and Sánchez-Villagra 2009; Wilson et al. 2010).

An early ossification of the scapula in relation to the humerus was retrieved for Xenarthra using Parsimov including ties (Fig. S4), but not with sequence-ANOVA. Even if the latter detected a significant heterochronic shift for the scapula by comparing developmental sequences between xenarthrans and marsupials, no significant difference was found between xenarthrans and other placentals. Because the early ossification of the scapula is one of several events tied at #1 in our sample, its apparent shift is likely a methodological artifact (Harrison and Larsson 2008). Otherwise, we did not detect any heterochronic shifts using Parsimov for xenarthrans at either the ordinal or subordinal level.

Although we used the same data as previous authors for all mammals but xenarthrans, our findings show varying levels of agreement with previous results. For instance, Sánchez-Villagra et al. (2008) found mammals to be characterized by a late development of the pterygoid relative to parietal, maxilla, and dentary, whereas we reported here no movement for the Theria clade (Figs. S2 and S3). For postcranial elements, we recover most of the results of Weisbecker et al. (2008). However, we found a new synapomorphy for the Marsupialia clade involving a late movement of the metatarsals in relation to caudal vertebrae and ischium (Fig. S4). Among

Euarchontoglires, the earlier onset of ossification of the periotic with respect to the palatine, basioccipital, basisphenoid and alisphenoid, considered by Sánchez-Villagra et al. (2008) as a potential human autapomorphy, was retrieved here, although we can add an early ossification of the Exoccipital in relation to the frontal, parietal, and jugal (Fig. S2). Concerning Rodentia, our results (Figs. S4 and S5) are highly incongruent with the findings of Wilson et al. (2010) and Weisbecker et al. (2008). We believe this is due to the present addition of xenarthrans and the sensitivity of Parsimov to taxon sampling, as noted by Wilson et al. (2010). The divergence between our results and those of previous studies may be due to the use of different parameters in running a Parsimov analysis (e.g., unrooted vs. rooted trees, considering ties or not), different outgroups (Wilson et al. 2010), the number of species sampled in a clade, the number of ties, or the addition of an entire placental groups (i.e., xenarthrans) that had not been included in previous studies.

Using sequence-ANOVA on postcranial sequences of all mammals but xenarthrans, we detected 12 significant differences between marsupials and placentals (Fig. 5). Among these, three were highly significant: the late ossification of the tibia, fibula, and femur of marsupials relative to placentals. These three changes were the only ones found by running Parsimov in our analysis with ties retained and in a previous study (Weisbecker et al. 2008). In that particular case, Parsimov was only able to detect highly significant differences. Thus, we view our results obtained with Parsimov by considering the strict consensus of ACCTRAN and DELTRAN optimizations as less reliable than those obtained by sequence-ANOVA, especially considering that most of the shifts detected by sequence-ANOVA were retrieved by Parsimov when either ACCTRAN and DELTRAN analyses were used alone, but not a consensus of the two (Figs. S2–S5). Only the early ossification of the manual and pedal phalanges was detected in the consensus of ACCTRAN and DELTRAN for the two performed analyses (considering ties or not), but solely for *Dasypus*. Because the ossification sequence of *Dasypus* is by far the most complete among our sample of xenarthrans, this result could also demonstrate the importance of using well-resolved sequences for performing Parsimov analyses.

Sequence heterochrony in xenarthrans

Our results show that heterochrony has played a role in the early skeletal development of xenarthrans. Although they have much in common with the developmental trajectory of other placentals, (Sánchez-Villagra 2002; Bininda-Emonds et al. 2003; Sánchez-Villagra et al. 2008; Weisbecker et al. 2008; Wilson et al. 2010), there are some significant differences: the late ossification of the sternum, and an early ossification of the pubis, manual, and pedal phalanges (Fig. 4).

No genuine heterochronic shifts for cranial elements were retrieved for xenarthrans at an ordinal or subordinal level. However, while without a strong significance level and possibly influenced by the accumulation of ties, our results are consistent with the interpretation of Schneider (1955) that the basicranium of *Bradypus* ossifies early. Furthermore, we note that this heterochrony is not shared by *Dasypus* or *Tamandua* (Figs. 3, S2, and S3). Our sequence analysis for cranial elements of the three-toed sloth (*Bradypus*) agrees with Schneider (1955) by showing a late ossification of the palatine, exoccipital, alisphenoid, basisphenoid, and basioccipital and an early ossification of the squamosal, pterygoid, and jugal (Fig. 3). Only a late ossification of the palatine compared to the premaxilla and nasal was retrieved for *Bradypus* by Parsimov.

It is also worth noting that all elements of the basicranium (basioccipital, exoccipital, basisphenoid, and to a lesser extent periotic) were characterized by delayed ossification, whereas the ossification of the squamosal, pterygoid, jugal, and lacrimal was always advanced. These observations may suggest the heterochronic shifts conform to the boundaries of modules previously identified for the mammalian cranium (Goswami 2006). Previous comparative analyses of cranial modularity have failed to identify coordinated heterochronic shifts in ossification timing that reflect phenotypic modules defined by morphometric data (Goswami 2007; Goswami et al. 2009). While sequence resolution was greater for many of the taxa included in those previous studies, no afrotherian or xenarthran placentals were included, as no comparative data from these taxa were previously available. The results of the analysis presented here suggest that xenarthrans may show a greater correspondence between phenotypic and developmental modules in the cranium, in contrast to the pattern observed in boreoeutherians or marsupials. Further analyses of modularity incorporating this new material, as well as data from afrotherians, are needed to explicitly test the relationship between cranial modularity and developmental timing in Atlantogenata in comparison to other mammals. Such studies may profit from dividing elements into modules based on developmental origin and growth.

Xenarthrans displayed many differences relative to marsupials (Fig. 4B). In agreement with the results of Weisbecker et al. (2008) for boreoeutherian placentals, they displayed an early ossification of the tibia, fibula, and femur relative to marsupials (Fig. 4B). The sequence-ANOVA showed that xenarthrans also differ from marsupials by a late ossification of the radius, sternum, cervical, and thoracic vertebrae, and an early ossification of the ilium, metatarsals, and pubis. Some of these shifts (late ossification of the sternum, cervical, and thoracic vertebrae, and the early ossification of the tibia,

fibula, and femur) were shown to characterize placentals relative to marsupials, with Parsimov analysis suggesting that the heterochronic changes occurred in the lineage leading to marsupials (Fig. 5, Weisbecker 2011).

Perhaps the most conspicuous difference of xenarthrans relative to other placental groups concerns the delayed ossification of the sternum. In contrast, ossification of the pubis, pedal, and manual phalanges is always advanced. Two of the four significant postcranial heterochronies that characterize xenarthrans relative to boreoeutherian placentals (Fig. 4A) involve elements of the limb girdles (sternum and pubis); two significant differences involve elements of the limbs (pedal and manual phalanges). The significance of these heterochronic shifts remains unclear, but they could be related to morphological particularities of Xenarthra. The late ossification of the sternum could be related to the fact that xenarthrans are unique among mammals by displaying ossified sternal ribs (Gaudin 2003). Accordingly, we did not observe any center of ossification in this part of the skeleton within our dataset of 74 fetuses and stillborns. Hypothetically, the early ossification of the pubis could be linked to the relative "sacralization" of the lumbar and posterior thoracic vertebrae in xenarthrans (MacPhee 1994), but no other heterochronic shift was detected for the remaining bones of the pelvic girdle.

Among placental mammals, the detection of a significant, early ossification of the pedal and manual phalanges (Fig. 4A) appears more striking. Heterochronic shifts involving the distal phalanges were also detected by the Parsimov analyses, but only for *Dasypus* (Figs. S4 and S5) and *Bradypus* and *Choloepus* assuming ACCTRAN (Fig. S4). The first elements to ossify for each digital ray are the distal phalanges; the medial and proximal phalanges ossify much later. Xenarthrans are characterized by long, sharp, and strong claws (Nowak 1999) that are associated with an enlargement of their distal phalanges. Huxley (1932) proposed that the time of initiation of an organ is related to its adult size. This appears to apply to the distal phalanges of xenarthrans, although other mammals (e.g., *Talpa europaea*) have enlarged digits without early ossification (Prochel 2006; Prochel et al. 2008). Moreover, this enlargement mainly concerns manual phalanges, while pedal phalanges—especially of anteaters and armadillos—are smaller. Interestingly, xenarthrans resemble marsupials in terms of the timing of phalangeal ossification (Fig. 4b), and are the only placental mammals to show such an early ossification of the manual phalanges. Weisbecker (2011) recently showed that monotremes (i.e., *Ornithorhynchus* and *Tachyglossus*) are also characterized by an early ossification of the phalanges. This heterochronic shift therefore appears to distinguish xenarthrans from other placental mammals. Furthermore, it could be considered as a plesiomorphic feature present in monotremes, marsupials, and xenarthrans, consistent with the Epitheria hypothesis in which xenarthrans are the basal-most placental mammals (McKenna 1975; Kriegs et al. 2006).

As coded in our analysis, not a single significant heterochronic shift involved the vertebral column at the node joining xenarthrans to other mammals. Nonetheless, compared to other mammals, vertebrae of xenarthrans appear very distinctive in displaying supernumerary articulations, termed "xenarthrales," on the lumbar and the thoracic vertebrae (Gaudin 1999). We observed no indication of an early ossification of these typical zygapophyseal articulations in our dataset, and we presume they ossify at a relatively late stage. Moreover, Hautier et al. (2010) have recently shown that the neural arches and centra of the vertebrae display different patterns of ossification. Compared with placental mammals, *Bradypus* shows an early ossification of the centra relative to the neural arches of its vertebrae. Future studies may benefit from coding ossification centers within elements to increase the sensitivity of an analysis, enabling detection of more subtle heterochronies than those we have identified by coding entire bones.

CONCLUSIONS

This developmental study presents the largest dataset provided to date for xenarthrans. We have found more inconsistency with previous studies using Parsimov than with those using the sequence-ANOVA method developed by Nunn and Smith (1998). The latter method represents a reasonable alternative that allowed us to detect ossification heterochronies in xenarthrans relative to other groups of mammals that would otherwise have remained invisible. Both methods are subject to type II errors due to the accumulation of ties at early events, which artifactually elevate the "significance" of early shifts due to low resolution of the earliest developmental events. It is nevertheless possible to control for such artifacts by weeding out heterochronies that occur among a series of early, tied events, and by comparing results from sequence-ANOVA with those from Parsimov assuming either ACCTRAN or DELTRAN.

With these qualifications, xenarthrans show a substantial degree of developmental distinctiveness compared to other placentals. Relative to northern placental mammals, we infer heterochronies in xenarthrans concerning the late ossification of the sternum, and an early ossification of the pubis, pedal, and manual phalanges. In addition to differences observed in patterns of dental eruption (Asher et al. 2009) and vertebral variability (Asher et al. 2011), these differences provide more evidence for inferring a dichotomy within placental mammals, a possibility that now must be tested with data on the skeletal development of afrotherians. Such study will allow better recognition of the morphology of the common ancestor from which all placental mammals have evolved.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. List of studied specimens. *Abbreviations*: ZMB, Museum für Naturkunde Berlin; MNHN, Muséum National d'Histoire Naturelle in Paris; BMNH, Natural History Museum London; UMZC, Museum of Zoology Cambridge; IRSNB, Institut Royal des Sciences Naturelles de Belgiques; VH, Vera Weisbecker personal collection.

Fig. S2. Parsimov results for the cranial dataset with all data coded, including ties. Elements in bold face are common to both ACCTRAN and DELTRAN reconstructions.

Fig. S3. Parsimov results for the cranial dataset with ties recoded as missing data. Elements in bold face are common to both ACCTRAN and DELTRAN reconstructions.

Fig. S4. Parsimov results for the postcranial dataset with all data coded, including ties. Elements in bold face are common to both ACCTRAN and DELTRAN reconstructions.

Fig. S5. Parsimov results for the postcranial dataset with ties recoded as missing data. Elements in bold face are common to both ACCTRAN and DELTRAN reconstructions.