doi:10.1111/j.1558-5646.2008.00424.x

OSSIFICATION HETEROCHRONY IN THE THERIAN POSTCRANIAL SKELETON AND THE MARSUPIAL–PLACENTAL DICHOTOMY

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Received February 21, 2007
Accepted April 25, 2008

Postcranial ossification sequences in 24 therian mammals and three outgroup taxa were obtained using clear staining and computed tomography to test the hypothesis that the marsupial forelimb is developmentally accelerated, and to assess patterns of therian postcranial ossification. Sequence rank variation of individual bones, phylogenetic analysis, and algorithm-based heterochrony optimization using event pairs were employed. Phylogenetic analysis only recovers Marsupialia, Australidelphia, and Eulipotyphla. Little heterochrony is found within marsupials and placentals. However, heterochrony was observed between marsupials and placentals, relating to late ossification in hind limb long bones and early ossification of the anterior axial skeleton. Also, ossification rank position of marsupial forelimb and shoulder girdle elements is more conservative than that of placentals; in placentals the hind limb area is more conservative. The differing ossification patterns in marsupials can be explained with a combination of muscular strain and energy allocation constraints, both resulting from the requirement of active movement of the altricial marsupial neonates toward the teat. Peramelemorphs, which are comparatively passive at birth and include species with relatively derived forelimbs, differ little from other marsupials in ossification sequence. This suggests that ossification heterochrony in marsupials is not directly related to diversity constraints on the marsupial forelimb and shoulder girdle.

KEY WORDS: **Event pair, heterochrony, life history, neonate, pouch, Theria.**

Heterochrony, or change of developmental timing or growth rates, is an important parameter in the evolution of morphological diversity (Gould 1977; McKinney and McNamara 1991; Richardson 1999). It is a developmental mechanism through which the evolution of morphological traits can be altered (e.g., Gould 1977; Hall 2003; McNamara and McKinney 2005). Heterochrony often coincides with changes in life-history parameters because these expose the growing organism to differing selection pressures (Bernardo 1993; McKinney and Gittleman 1995; Ryan and Semlitsch 1998).

Mammalian postcranial evolution has been discussed as an example of how marked disparity in morphological diversity can result from differences in life history (Müller 1967; Lillegraven 1975; Sears 2004). The mammalian postcranial skeleton displays an impressive array of diverse phenotypes, including adaptation for flight in bats, for obligate swimming in cetaceans, pinnipeds,

and sirenians, and for fossoriality in many clades. However, most of this diversity occurs in placentals. Marsupials, who are also less speciose (Nowak 1999), show comparatively little morphological diversity in their postcranial skeleton, particularly with respect to their forelimbs (Lillegraven 1975; Sears 2004). This contrast between marsupials and placentals coincides with extensive differences in life-history traits relating to neonatal maturity and gestation length (Lillegraven 1975, 1979). Whereas marsupials have a uniformly short gestation period varying less than fourfold—from 12.5 days reported in two peramelemorphs (Strahan 1997) to 45 days in Matschie's tree kangaroo (Flannery 1995)—placentals have longer gestation periods that vary more extensively (roughly 40-fold; 16 days in the hamster *Mesocricetus auratus* and over 660 days in elephants; Nowak 1999). Marsupial neonates are too immature to survive away from the mother; following birth, they immediately move toward the teat (Gemmell et al. 2002; Tyndale-Biscoe 2005). This movement is achieved by action of the shoulder girdle and/or forelimb, which are conspicuously developed and appear specially adapted for climbing motion (Klima 1987; Sánchez-Villagra and Maier 2003). It has been suggested that the combination of extreme altriciality and functional demands of the postnatal move places a constraint on the phenotypic variation of the marsupial forelimb (Lillegraven 1975; Gemmell et al. 2002; Sears 2004; but see Kirsch 1977). This hypothesis is supported by a study on marsupial scapular and pelvic diversity (Sears 2004), which demonstrated that marsupial scapular shape is less diverse and ontogenetically more homogenous than that of placentals. As a mediator for this diversity constraint, heterochronic acceleration of forelimb development in marsupials compared to placentals is often named (Gemmell et al. 1988; Frigo and Wooley 1996; Sears 2004). However, empirical support for this proposal has only recently emerged through comparisons of chondrification and ossification sequences in the two clades (Sánchez-Villagra 2002; Bininda-Emonds et al. 2007). Developmental sequences are suitable for investigating the influence of evolutionary change on ontogeny (Smith 1997; Bininda-Emonds et al. 2002; McNamara and McKinney 2005); therian postcranial ossification and chondrification patterns are of particular interest because they occur before (in the case of chondrification) or soon after (in the case of ossification) birth in marsupials (e.g., Hall and Hughes 1987; Gemmell et al. 1988; Frigo and Wooley 1996; de Oliveira et al. 1998). Using event-pair analysis (where the timing of each event is related to every other event), the studies by Sánchez-Villagra (2002) and Bininda-Emonds et al. (2007) demonstrated that chondrification and ossification onset timing of fore-and hind limbs is indeed heterochronic between placentals and marsupials. These results are congruent with the hypothesis that marsupial forelimb development is accelerated compared to that of placentals, but the direction and polarity of this heterochrony has not been established. Consequently, it is also possible that it is the placentals whose skeletal formation is heterochronic, suggesting that the developmental sequence of marsupials is plesiomorphic; alternatively, it could be the marsupial hind limb that develops late, implying that heterochrony between marsupials and placentals is more complex than previously thought. The latter scenario has already been proposed by Müller (1967), although this part of her work was never discussed elsewhere (perhaps because Müller's papers were written in German). In the absence of a close outgroup (monotremes have not been sampled due to lack of available material), the direction and character of sequence heterochrony in the limbs of marsupials and placentals can only be confirmed using the remaining postcranium as a reference. Although Sánchez-Villagra's (2002) study included ossification onset timing of the whole postcranium, the sample size was small and interpretation of heterochrony and its polarity in event-pair analysis was limited because of the complexities of summarizing the large datasets resulting from event-pair coding (Jeffery et al. 2005). Parsimov, a computer program developed by Jeffery et al. (2005), can improve this by implementing an algorithm that analyses all possible scenarios of heterochrony and returns the most parsimonious solution (i.e., that involving the least heterochrony).

The present study includes postcranial ossification data from 11 marsupial and 13 placental species, a tripling of the taxonomic sampling from Sánchez-Villagra's (2002) dataset. Improved taxonomic representation provides a more reliable basis on which hypotheses of ossification heterochrony between marsupials and placentals can be tested. Large-scale sampling also allows additional comparison between marsupials and placentals with respect to the variation in ossification sequence positions of single bones. We examine questions pertaining to the potential influence of the marsupial/placental life-history dichotomy on postcranial skeletal ontogeny: Is the forelimb of marsupials really accelerated compared to that of placentals? Are differences between marsupials and placentals also reflected in variations of ossification sequence ranks of single bones? Lastly, is heterochrony common within marsupials and placentals, and does it provide any phylogenetic signal?

Material and Methods

DATA ACQUISITION

Twenty-five ossification events for a range of species were recorded from the literature as listed in Table 1. New data were obtained for nine marsupial species and three placental species (for accession numbers, see Appendix I). Care was taken to evenly sample specimens of different sizes to prevent under-sampling of the sequence; however, very young specimens of *Petaurus* were unavailable. Most placental data were collected using clearing and staining of developmental series (Prochel 2006). The majority of marsupial specimens was sampled by borrowing pouch-young

Table 1. Species names, specimen and stage number, and references of specimen data taken from the literature.

Species name	Specimen numbers/stages	Reference
Chelydra serpentina	47/7	Rieppel 1993b
Alligator misssissippiensis	36/9	Rieppel 1993a
Lacerta vivipara	23/8	Rieppel 1993b
Myotis lucifugus	19/7	Adams 1992
Homo sapiens	60/17	Mall 1906
Rattus norvegicus	N.a./14	Strong 1925
Mus musculus	41/5	Johnson 1933; Patton and Kaufman 1995
Mesocricetus auratus	168/8	Beyerlein et al. 1951
Meriones unguiculatus	187/8	Yukawa et al. 1999
Bos taurus	180/9	Lindsay 1969a, b
Sus scrofa	N.a./12	Stöckli 1922
Talpa europaea	22/9	Prochel 2006
Cavia porcellus	N.a./12	Petri 1935
Didelphis virginiana	16/9	de Oliveira et al. 1998
Sminthopsis macroura	11/8	Frigo and Wooley 1996

specimens from museum collections and assessing the presence of bone through acquisition of shadow images (comparable to a conventional high-resolution x-ray) in a SkyScan 1172 desktop micro-CT scanner (Skyscan, Kontich, Belgium). Shadow images were taken at 30°—angles throughout 360° rotation, using low voltage and high current settings (59 kV/167A) to improve detection of the relatively thin bone. In all but the smallest specimens, the upper and lower halves of the body were separately acquired to allow for greater magnification. 3D-visualizations of selected specimens were also conducted using VGStudioMax software (ver. 1.2; Volume Graphics, Heidelberg, Germany), but all scoring was conducted based on shadow images. In two marsupial species (*Isoodon macroura* and *Trichosurus vulpecula*), a combination of clear-staining results and CT-shadow imaging were employed; in these cases, the results were checked in detail to ensure that no conflict existed in the ossification sequence data. In the case of the genera *Petaurus* and *Dasyurus*, "chimaera" sequences of two closely related species were investigated; again, no conflicts between ossification sequences were detected. The sequence of ossification onset was noted in every species; epipubic bones, which existed in the earliest placentals (Ji et al. 2002) but were subsequently lost, were scored to occur last in placentals. Sampling 27 species for 25 events resulted in a dataset of 675 events, of which only seven could not be determined.

The use of clearing and staining as well as x-ray to obtain data from placental and marsupial specimens merits brief comment. It is possible that bones that are at very early stages of ossification would be detected earlier by one technique compared to the other due to potentially different sensitivities of the methods, as is the case also when comparing histological data with those derived from whole mount preparations (Sánchez-Villagra 2002). This can lead to different scores of bone presence in specimens with the same degree of ossification. However, although failure to detect weak ossification will only delay the detection of ossification onsets, it will not lead to the coding of different sequences.

An event-pair matrix was constructed based on ossification sequences by relating the ossification onset of each element to every other (Smith 1997). If one element appeared before another, this event pair was given a score of 0; if the two elements appeared simultaneously, the score was 1, and if an element appeared after another, the event pair was given a score of 2. A matrix of $\frac{1}{2}$ $(25^2 - 25) = 300$ events was thus scored following the scoring direction of column versus row events given by Jeffery (2005).

OSSIFICATION RANK ANALYSES

The raw ossification onset data for every species were converted to ranks (e.g., element to ossify first, second. . .), with ossifications reported at the same time receiving the same rank. To ensure that low specimen sampling did not influence the resolution of ranks, the maximum number of ranks was also regressed against sample size for each species. Because extremely large samples are not expected to improve resolution, species with very large sample sizes were assigned a sample size of 60 (which is the sampling of the best resolved species, *Homo*) to avoid high leverage datapoints. To address the question as to whether rank variation of individual bones is different across marsupials compared to placentals, ossification onset ranks were standardized by the maximum number of ranks (r_{max}) in every species so that the ranks are distributed between $1/r_{max}$ and 1. This creates some additional noise because species with high r_{max} have a lower influence on the variance, but the difference between maximum ranks necessitated this scaling. Rank ranges of each bone across marsupials and placentals were determined and compared.

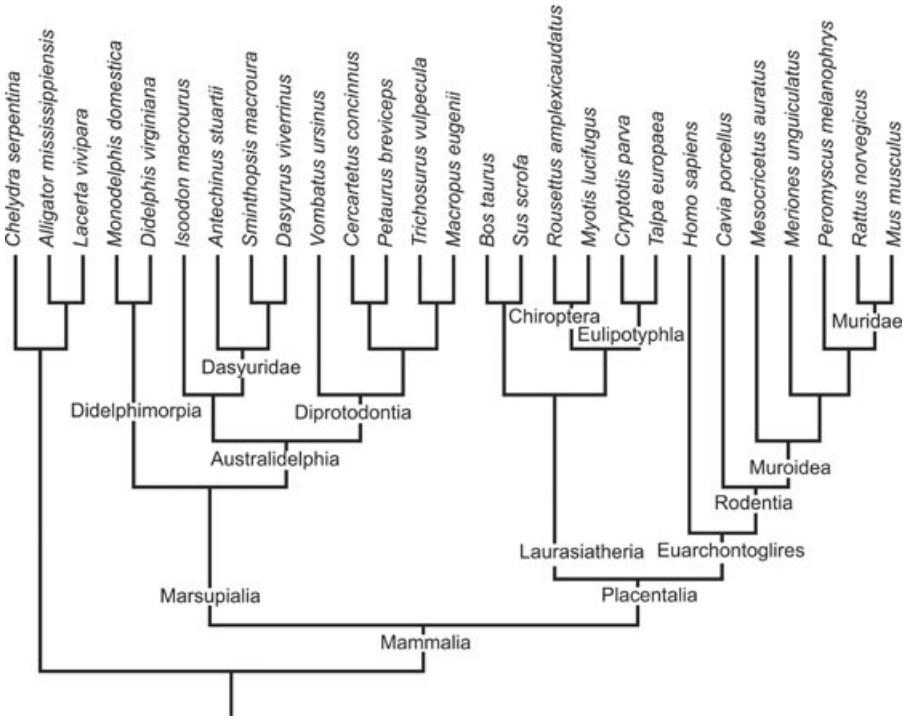

Figure 1. Phylogeny of all species examined in this analysis, with taxonomic of names major clades.

PHYLOGENETIC ANALYSIS

Although event-pair data are arguably unsuitable for phylogenetic analysis due to issues of nonindependence (Bininda-Emonds et al. 2002; Schulmeister and Wheeler 2004), parsimony analysis can be used to broadly assess the phylogenetic signal of event-pair data (Sánchez-Villagra 2002). Phylogenetic analysis is also a convenient way of obtaining an overall impression of heterochrony occurring between clades and the events involved. Parsimony analysis of event-pair data was conducted using PAUP* version 4.0b10 (Swofford 2001). It was impossible to retain the arrangement of the outgroup taxa during PAUP analysis because *Chelydra* is highly divergent. Therefore, only an outgroup comparison with *Lacerta* and *Alligator* was run.

PARSIMOV ANALYSIS

Event-pair analyses were conducted using a compound phylogeny (Fig. 1). Marsupial interordinal relationships reflect most of the latest phylogenies (e.g., Asher et al. 2003; Cardillo et al. 2004; Nilsson et al. 2004), relationships within Dasyuromorpha were based on Krajewski et al. (2000), and relationships within Diprotodontia, with paraphyletic possums, are according to most of the latest phylogenetic analyses (Osborne et al. 2002; Phillips et al. 2006; Meredith et al. 2007; Beck 2008). Interordinal relationships between placentals were based on Springer et al. (2004) and relationships within rodents follow Steppan et al. (2004). The outgroups are arranged following Zardoya and Meyer (2001), Mickoleit (2004), and Scheyer (2007). The Parsimov program package (Jeffery et al. 2005) was employed for computer-based analysis of heterochrony. Parsimov is a program that uses apomorphy lists derived from parsimony-based phylogenetic analysis to explain as many sequence shifts as possible with as little heterochrony as possible. Jeffery et al. (2005) recommend running Parsimov on ACCTRAN and DELTRAN optimizations and only considering the strict consensus of the two. Because the single ACCTRAN and DELTRAN-based results are already a strict consensus of runs with different input orders (Jeffery et al. 2005), the Parsimov approach is conservative but also reliable. It should therefore be noted that what is reported here is an estimate of minimal heterochronic changes.

Phylogenetically based event-pair analysis has been criticised for the use of temporally and biologically nonindependent data in a phylogenetic framework and it has been shown that this approach can lead to results that are not supported by the original data (Schulmeister and Wheeler 2004). All relevant results were therefore double-checked by mapping event pairs for which heterochrony was suggested on the phylogeny.

Results

OSSIFICATION SEQUENCES AND RANK VARIATION

The ranked list of ossifications is presented in Table 2. Across all species, the number of ranks was generally much lower than the number of specimens investigated, and no intraspecific variation in ossification sequence was found in any species. Frequency

Table 2. Ranked ossification onsets, and number of specimens, for all species examined.

	Chelydra serpent.	*Alligat. mississ.*	*Lacerta vivipara*	*Myotis lucifug.*	*Homo sapiens*	*Bos taurus*	*Sus scrofa*	*Rattus norveg.*	*Mus muscul.*	*Merion. unguicu.*	*Peromy. melano.*	*Talpa spp.*	*Cryptotis parva*
Number of specimens	47	36	23	19	60	180	n.a.	n.a.	41	187	7	22	15
Clavicle	1	?	1	1	1	1	?	1	1	1	1	1	1
Humerus	2	1	1	2	2	2	1	2	2	2	2	3	3
Ribs	2	4	3	3	5	3 or 4	3	2	2	?	2	1	2
Femur	2	1	1	2	2	2	1	3	2	2	2	3	3
Radius	3	1	1	2	3	3	1	2	2	2	2	2	3
Ulna	3	1	1	2	1	3	1	2	2	2	2	2	3
Scapula	3	4	3	3	5	3	2	3	2	2	2	2	3
Cervic.	3	3	3	3	7	4	4	3	2	2	2	2	3
Thorac.	3	4	5	3	7	4	4	3	2	3	2	2	3
Tibia	3	1	1	2	3	3	1	3	2	2	2	2	3
Fibula	3	1	1	2	5	3	2	3	2	2	2	2	3
Lumbar	3	4	5	3	9	3 or 4	4	4	2	3	2	4	4
Sacral	3	5	5	6	11	3 or 4	4	4	3	4	2	6	4
Caudal	3	5	6	6	14	5	6	4	3	6	3	7	5
Ilium	4	6	3	3	6	4	2	3	2	3	2	5	4
Man. phal.	4	7	3	5	6	4	5	7	3	5	3	8	4
Ped. phal.	4	7	2	3	8	4	7	7	4	5	3	8	6
Ischium	5	6	7	5	12	4	8	5	3	5	3	8	4
Pubis	5	7	4	5	15	8	11	5	3	8	3	8	6
Metac	6	5	3	3	7	4	5	4	3	4	3	8	6
Metat	6	2	2	4	8	4	7	4	3	5	3	8	7
Tarsals	6	9	6	6	10	7	9	6	4	7	4	8	7
Carpals	7	8	8	7	16	9	12	8	5	8	4	9	7
Sternum	8	10	9	3	13	6	10	5	3	?	3	6	4
Epipubics	8	10	9	8	17	10	13	9	6	9	5	10	8
Total Ranks[1]	7	9	8	7	16	9	12	8	5	8	4	9	7

Continued

Table 2. Continued

	Rousett. amplex.	*Mesocri. auratus*	*Cavia porcell.*	*Monod. domest.*	*Didelp. virgin.*	*Sminth. macrou.*	*Dasyur. viverr.*	*Antech. stuartii*[2]	*Isoodon macro.*	*Cercart. concinn.*	*Trichos. vulpec.*	*Macrop. eugenii*	*Petaurus brevic.*	*Vombat. ursinus*
Number of specimens	12	168	n.a.	13	16	11	20	23	15	25	32	11	23	10
Clavicle	1	1	1	1	1	1	1	1	1	1	2	1	1	1
Humerus	1	2	2	2	2	1	1	1	1	1	2	1	1	1
Ribs	1	2	3	2	2	2	1	1	1	1	2	1	1	1
Femur	1	3	2	5	5	3	2	2	3	2	3	2	1	2
Radius	1	3	2	2	2	1	1	1	1	1	2	1	1	1
Ulna	1	3	2	2	2	1	1	1	1	1	2	1	1	1
Scapula	1	3	3	2	2	1	1	1	1	1	2	1	1	1
Cervic.	3	3	4	2	2	2	1	1	1	1	2	1	1	1
Thorac.	5	3	5	3	2	2	1	1	1	1	2	1	1	1
Tibia	2	3	2	5	5	3	2	2	3	2	3	2	1	2
Fibula	2	3	2	5	5	3	2	2	3	2	3	2	1	2
Lumbar	5	3	5	4	3	3	2	2	4	2	3	2	1	2
Sacral	7	4	8	5	5	3	2	3	5	2	4	4	1	2
Caudal	7	6	10	6	5	4	3	3	5	3	4	4	2	3
Ilium	5	3	4	5	5	3	2	2	4	2	4	2	1	2
Man. phal.	6	4	6	2	5	3	3	3	2	2	1	1	2	1
Ped. phal.	6	4	7	8	6	5	5	7	6	2	3	3	2	2
Ischium	7	6	5	7	6	3	4	3	4	4	4	2	2	3
Pubis	6	6	10	8	7	6	8	8	6	7	8	7	3	5
Metac	4	4	6	6	4	3	3	4	2	4	4	3	1	2
Metat	7	6	7	8	6	4	5	6	6	5	6	6	3	4
Tarsals	8	7	11	9	8	7	9	9	8	6	7	8	4	4
Carpals	10	8	12	10	8	8	10	10	10	7	9	9	5	6
Sternum	9	5	9	8	6	5	6	5	7	5	6	6	3	4
Epipub.	11	9	13	5	9	4	4	6	9	8	5	5	6	4
Total no.	10	8	12	5	9	8	10	10	10	8	9	9	6	6

[1]Due to assignment of the last ranks to events that never appear (e.g., epipubics), the number of actually observed ranks may be lower than the maximum rank number.

[2]Pubis, Tarsals, and carpals could not be determined in *A. stuartii* but because their order is the same in all other marsupials, they were scored in succession

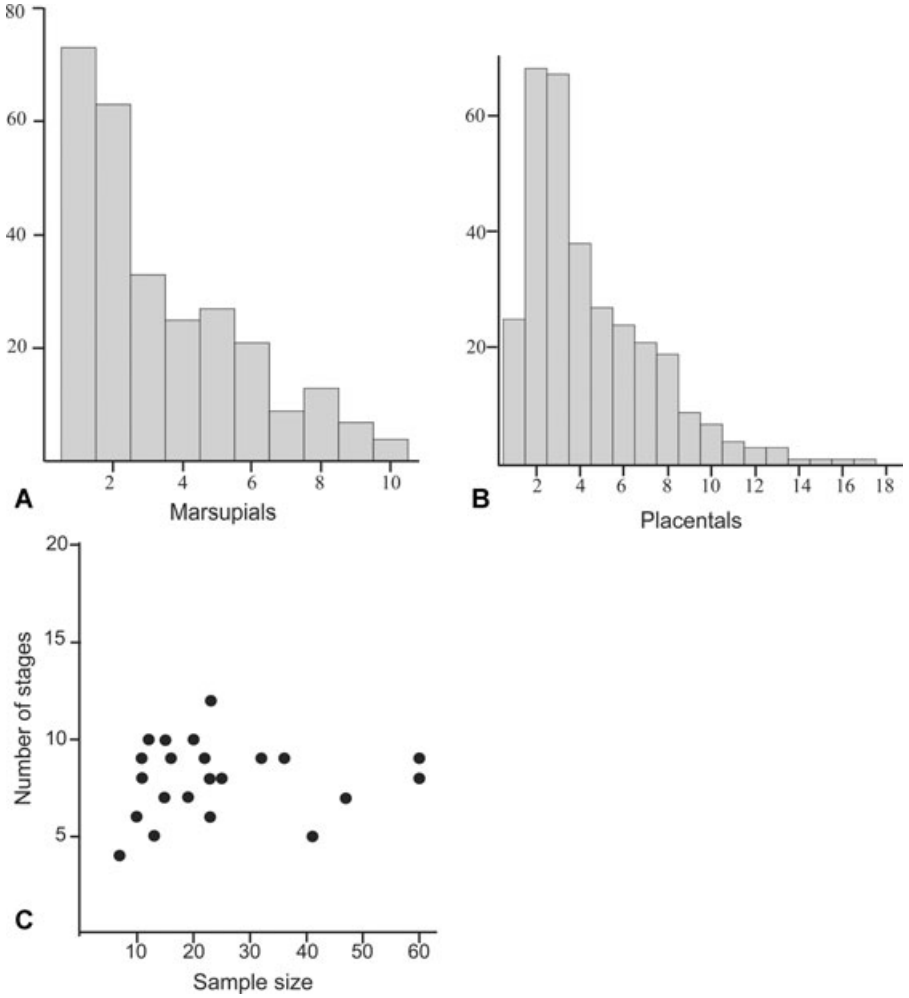

Figure 2. Frequency distribution of ranks in all marsupial (A) and all placental (B) species and plot of samples sizes versus number of stages (C).

distributions of ranks (Fig. 2A,B) showed that ossification event occurrences are skewed toward earlier stages of ossifications in most mammals; this is particularly the case in marsupials (Fig. 2A), where many postcranial elements ossify simultaneously in the first few ranks. The correlation between the number of specimens and number of ranks was not significant ($r = 0.309$; $P = 0.141$; Fig. 2C), suggesting that specimen numbers are not affecting the rank resolutions.

Rank variation of individual bones in marsupials and placentals is presented in Figure 3. Two areas of the postcranium reveal particularly conspicuous differences between the two clades; first, forelimb and tarsal/metatarsal rank variation is much lower in marsupials compared to placentals, and second, hind limb and posterior element (except for tarsal/metatarsals) rank variation is lower in placentals compared to marsupials.

PHYLOGENETIC ANALYSIS

Phylogenetic analysis of event pairs using PAUP resulted in 59 (19.6%) constant characters, 55 (18.3%) variable but parsimony-uninformative characters, and 186 (62%) parsimony-informative characters. The most parsimonious phylogenetic tree (Fig. 4) does not return much of commonly accepted phylogenetic relationships within placentals except for Eulipotyphla; Marsupialia and Australidelphia are also retrieved. The single exception is the glider *Petaurus*, of which very few early stages could be sampled; this lead to low resolution in the earlier ranks. Parsimony analysis groups *Petaurus* with other species with particularly few ranks. Marsupials are diagnosed by 31 apomorphies, 19 of which are related to heterochrony between the appearance of hind limb bones and bones anterior in the body (Appendix II); the remaining apomorphies mostly relate to sequence shifts between anterior and more posterior bones.

PARSIMOV ANALYSIS

The consensus results of heterochrony reported by Parsimov are presented in Table 3. Some placental clades are heterochronic, but no heterochrony is reported for the whole of Placentalia. Late movement of the hind limb long bone ossifications with respect

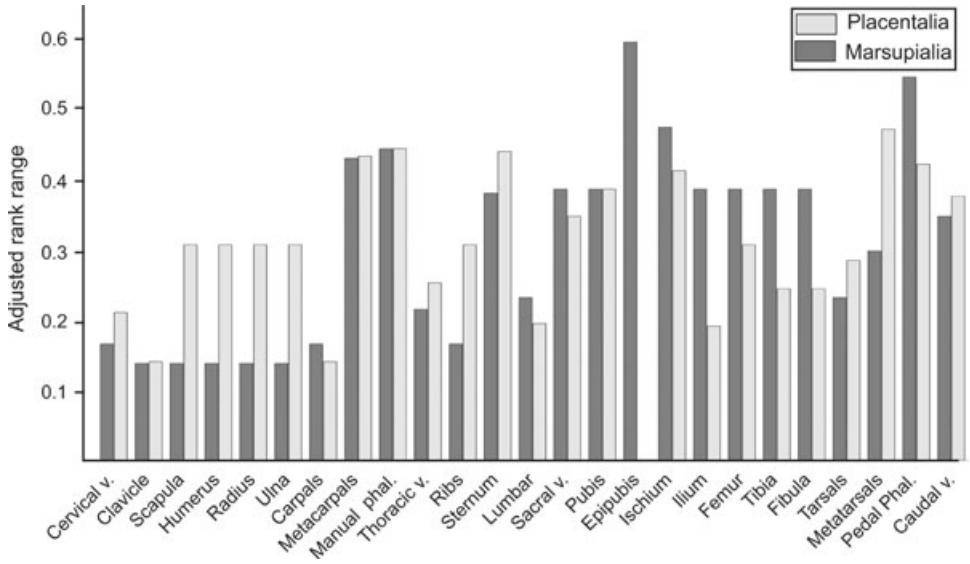

Figure 3. **Adjusted rank ranges of all bones examined, ordered from anterior to posterior in the body.**

to the ilium and forelimb long bones is reported in marsupials. An example of the disparity between ossification onset of anterior compared to posterior postcranium in marsupials is shown in Figure 5. This heterochrony appears to be specific to femur, tibia, and fibula, and does not reflect a more generalized posterior delay because late movement of the hind limb long bones is also relative to the nearby ilium (and in separate ACCTRAN and DELTRAN-reports also the thoracic and lumbar vertebrae). Examination of the results from the single ACCTRAN and DELTRAN analyses (detailed in Table 4) also provides support for late ossification of hind limb long bones with respect to more anteriorly located vertebral elements. In addition, more than 90% of the ACCTRAN runs support the DELTRAN report of a shift to early ossification of cervical vertebrae, ribs, and scapula with respect to the forelimb long bones in marsupials. It is notable that both late ossification of the hind limb long bones and early ossification of

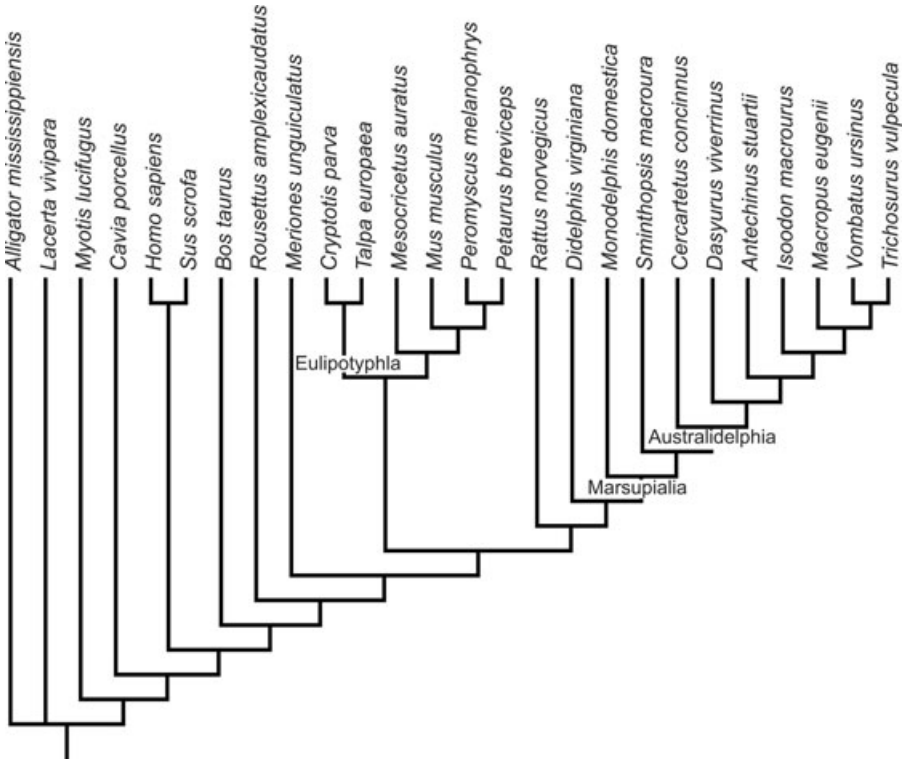

Figure 4. **Phylogeny obtained from parsimony analysis of event-pair scores, with clades named that are correctly retrieved.**

Table 3. Heterochronies in major mammalian clades as reported by the ACCTRAN and DELTRAN consensus from parsimov.

	Sauropsida	
Sternum	late	Carpals* Tarsals*, Pubis*
	Theria	
	No movement	
	Marsupialia	
Fibula	Late	Ilium, Ulna, Radius
Tibia	Late	Ilium, Ulna, Radius
Femur	Late	Ilium, Ulna, Radius, Humerus
	Ameridelphia	
Lumbar vertebrae	early	Tibia, Fibula, Femur
	Australidelphia	
Clavicle	late	Scapula, Cervical v., Ribs, Ulna, Radius, Humerus
	Peramelemorpha	
Pubis	early	Metatarsals, Sternum*, Pedal phalanges
Ilium	late	Ischium, Fibula, Tibia, Femur
Lumbar v.	late	Ischium
Metacarpals	early	Fibula*, Tibia*, Femur*
	Dasyuromorphia	
Manual phalanges	early	Ilium*, Tibia*, Fibula*, Femur*
Epipubis	early	Tarsals*, Metatarsals
	Diprotodontia	
	No movement	
	Placentalia	
	No movement	
	Laurasiatheria	
	No movement	
	Eulipotyphla	
Thoracic v.	early	Fibula, Ulna, Radius
Cervical v.	early	Fibula, Ulna, Radius
Ribs	early	Scapula, Femur*, Ulna*, Radius*
Metatarsals	early	Caudal v.*, Tarsals
	Scrotifera	
	No movement	
	Chiroptera	
	No movement	
	Artiodactyla	
	No movement	
	Euarchontoglires	
Ilium	early	Lumbar v., Thoracic v.*
	Homo	
Tarsals	early	Caudal v.*, Pubis*, Sternum*
Manual phalanges	early	Ilium, Metacarpals
	Rodentia	
	No movement	
	Muroidea	
Scapula	early	Tibia, Femur
Cervical v.	early	Tibia, Fibula, Femur, Ulna, Radius
	Muridae	
Sacral v.	late	Caudal v., Metatarsals
Pedal phalanges	late	Caudal v., Pubis, Ischium, Metatarsals, Sternum

***Note*: An asterisk (*) denotes heterochronies involving complete position shifts ("before–after" or "after–before").**

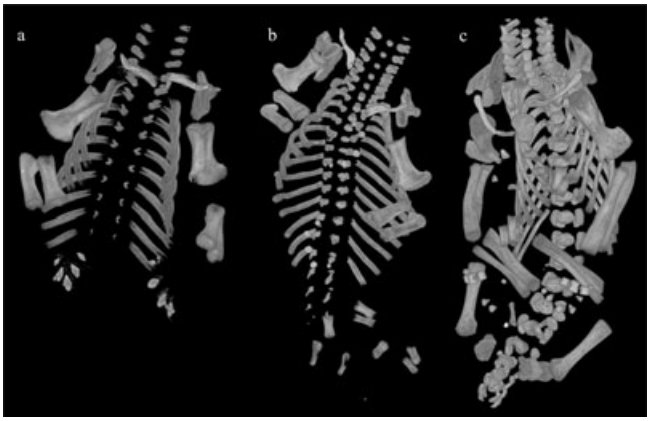

Figure 5. Three-dimensional reconstruction of CT-scans of postcranial bones in 1-day (A), 5-day (B), and 21-day (C) old pouch young Tammar wallaby (*Macropus eugenii*).

anterior axial elements are reported relative to the forelimb, whose ossifications are not reported to shift. Mapping of the characters suggested to be heterochronic by Parsimov reveals that the proposed sequence shifts between marsupials and placentals largely involve shifts toward or away from simultaneity (for examples, see Fig. 6).

Discussion

HETEROCHRONY AT THE MARSUPIAL–PLACENTAL DICHOTOMY

It has previously been assumed that marsupials differ from placentals through developmental acceleration of the forelimb as a result of early forelimb functionality; however, our results demonstrate that the differences in postcranial ontogeny between marsupials and placentals are more complex. This is already apparent from simple phylogenetic analysis; although little phylogenetic information is retrieved otherwise, differences relating to late ossification of the hind limb long bones and earlier ossification of anterior axial elements place nearly all marsupials into a monophyletic group. The conservative pattern of late ossification of hind limb long bones with respect to forelimb and shoulder girdle in marsupials is specifically identified as heterochronic by Parsimov. In addition, some anterior elements (scapula, cervical vertebrae, and ribs) are reported to shift from ossifying after the forelimb long bones to consistently ossifying at the same time, usually first or second in the sequence. The heterochronic divide between the anterior and posterior postcranium also coincides with changes of sequence position variation of single bones. Marsupials display greater sequence position variation of the hind limb long bones and greater position conservativity of the forelimb area. This coincidence of ossification sequence heterochrony and rank variation differences suggests a complex change of developmental patterns in the entire osseous postcranium of marsupials. Consequently we argue for a reassessment of the traditional hypothesis that the main difference between marsupial and placental postcranial development is advanced formation of the forelimb and shoulder girdles in marsupials.

Although prenatal developmental "dormancy" has been suggested for marsupial hind limb buds (Bininda-Emonds et al. 2007), the relatively small hind limb of marsupial neonates has usually been mentioned as emphasizing the strong forelimb development

Table 4. Detailed account of heterochronies between marsupials and placentals reported by Parsimov.

Moving event	moves. . .	. . .with respect to
ACCTRAN		
Manual Phalanges	Early	Lumbar Vertebrae, Metacarpals
Metatarsals	Late	Caudal Vertebrae, Sternum
Fibula	Late	Ilium, Lumbar vertebrae, Ulna, Radius
Tibia	Late	Ilium, Lumbar vertebrae, Ulna, Radius, Humerus
Femur	Late	Ilium, Lumbar vertebrae, Ulna, Radius
Epipubis	Early	Carpals, Pubis
DELTRAN		
Manual Phalanges	Early	Pedal phalanges, Ilium
Scapula	Early	Ulna, Humerus
Cervical vertebrae	Early	Ribs, Ulna, Radius, Humerus
Ribs	Early	Ulna, Radius, Humerus
Fibula	Late	Ilium, Thoracic vertebrae, Ulna, Radius
Tibia	Late	Ilium, Thoracic vertebrae, Ulna, Radius
Femur	Late	Ilium, Thoracic vertebrae, Ulna, Radius, Humerus
CONSENSUS		
Fibula	Late	Ilium, Ulna, Radius
Tibia	Late	Ilium, Ulna, Radius
Femur	Late	Ilium, Ulna, Radius, Humerus

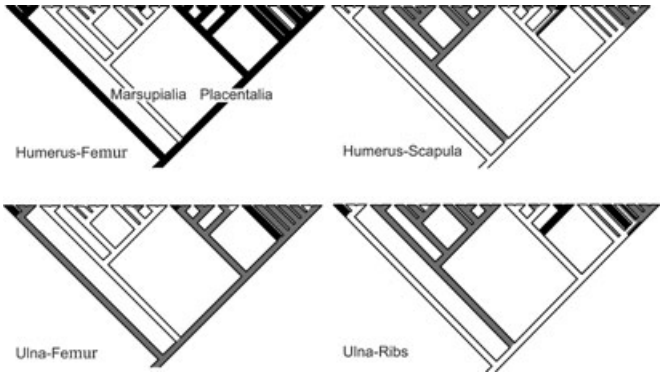

Figure 6. Some event pairs that distinguish marsupials from placentals mapped on an outline of the phylogeny used. The Ulna-Ribs event pair also distinguishes Eulipotyphla. White, first element ossifies before second; gray, elements ossify simultaneously; black, first element ossifies after second.

(Lillegraven 1975; Frigo and Wooley 1996; Maunz and German 1997; Bininda-Emonds et al. 2007), rather than as possibly heterochronic in relation to other body parts. However, Müller (1967) related the small size of the hind limb of marsupial neonates to the metabolic costs of extensive forelimb development to the growing embryo. This seems a reasonable proposition given the short gestation time available to the marsupial embryo, within which metabolic energy expenditure on the postcranium must guarantee the development of a functional forelimb at birth. This includes extensive prenatal development of soft tissues such as muscle, nerves, and blood vessels necessary to support the climb to the pouch (e.g., Sharman 1959; Frigo and Wooley 1996; Sánchez-Villagra and Maier 2003) and may occur at the metabolic "expense" of the hind limb until birth. Such energy allocation shifts related to strong perinatal soft-tissue development have also been postulated for the marsupial cranium by Smith (1997): as a result of the functional requirement for sufficient muscle to allow attachment to the teat and suckling, the central nervous system appears to develop late. It is conceivable that allocation of resources on the forelimb represents a second metabolic focus in the developing marsupial embryo, with the hind limb as another area of lower prenatal investment. As the time between birth and the first foray out of the pouch is considerably longer than gestation, data from previous studies suggest that marsupials compensate for any initial delays of the hind limbs through faster postnatal growth rate and longer growth of the hind limb compared to the forelimb (e.g., Guiler 1960; Lyne 1964; Merchant et al. 1984; Maunz and German 1997).

The heterochrony and ossification rank conservativeness involving marsupial cervical vertebrae, scapula, and ribs are well explained with the traditional model of early demands of forelimb functionality (e.g., Sharman 1959; Lillegraven 1975; Gemmell et al. 1988; Sánchez-Villagra 2002). The scapula is expected to ossify with the forelimb long bones because it is an integral part of the climb to the pouch (Klima 1987; Sánchez-Villagra 2002; Sánchez-Villagra and Maier 2003; Sears 2004). The ossification and specialized architecture of the forelimbs and shoulder girdle have been related to muscle-generated stresses of climbing (Klima 1987; Sánchez-Villagra and Maier 2003); given that the muscles suspending and moving the shoulder arch and humerus originate on the vertebral column and the ribs (e.g., trapezius, rhomboid, serratus, levator scapulae), ossification onset of ribs and cervical vertebrae just after birth is equally well explained as a consequence of muscular stresses.

Direct influence of muscle activity on ossification is reminiscent of the concept of "causal histogenesis" (Pauwels 1960), in which bone formation is influenced by mechanical impacts such as muscular stress (see also van der Klaauw 1946). Similar processes have been suggested based on observations on humans (the "mechanostat hypothesis"; Frost 1987; Rauch and Schoenau 2001), and it is known that muscular activity influences bone shape and formation during vertebrate bone ontogeny (e.g., Herring 1994; Rot-Nikcevic et al. 2006; Franz-Odendaal et al. 2007). It is therefore conceivable that ossification timing in the entire anterior region of marsupials is largely influenced by mechanical strains.

Our results suggest life-history-related heterochrony of ossification sequence in marsupials; however, the extent to which the factors that influence the postcranial development timing also constrain diversity of the marsupial forelimb cannot be directly established. Nevertheless, some insights may be gained from peramelemorphs (bilbies and bandicoots) and the marsupial mole genus *Notoryctes*, which include species with the most derived forelimbs among marsupials; peramelemorphs have also been shown to have deviant shoulder girdle growth patterns compared to other marsupials (Sears 2004). Although the ossification sequence of *I. macrourus* is largely similar to that of other marsupials (see also Gemmell 1988), the timing of postcranial ossification onset is relatively late at postnatal day 8 and the hind limb long bones ossify within two days after the forelimbs. In other marsupials, (*Didelphis virginiana*, *T. vulpecula*, *Sminthopsis macroura*, *Didelphis albiventris*; Nesslinger 1956; Gemmell et al. 1988; Frigo and Wooley 1996; de Oliveira et al. 1998) ossification commences earlier (just before to five days after birth) and the hind limb long bones ossify long after the forelimb long bones (after 5–7 days). Although all marsupials actively contribute to their placement into the pouch, *Isoodon macrourus* moves in a "snake-like wriggle" without using its forelimbs to climb (Gemmell et al. 2002); the marsupial mole *Notoryctes* may also reach the pouch with little climbing movement (Sears 2004). This mode of birth may have lifted the diversity constraints on the forelimb and shoulder

girdle due to comparative relaxation of postnatal functional requirements (Sears 2004). Should this be the case, the differently timed but conserved ossification sequence in *Isoodon* supports the hypothesis that specialized limb functionality at birth, rather than the processes leading to ossification sequence heterochrony, primarily govern diversity constraints on the marsupial forelimb and shoulder girdle.

HETEROCHRONY IN MORE-INCLUSIVE THERIAN MAMMAL CLADES

The heterochrony at the marsupial–placental dichotomy reported between marsupials and placentals is contrasted by general lack of heterochrony between mammals and sauropsids, and in most major placental clades. Where Parsimov identified heterochrony in previously studied clades (Sánchez-Villagra 2002; Prochel 2006), these were largely similar to the results of these studies. Among the few notable heterochronic shifts with phylogenetic signal is that uniting Eulipotyphla, represented by the mole *Talpa europaea* and the shrew *Cryptotis parva*. Early ossification of anterior postcranial elements has already been described for the mole *T. europaea* by Prochel (2006); the addition of *Cryptotis* to the analysis extends this shift of the anterior axial skeleton to all Eulipotyphla. This shift resembles that reported for marsupials; in fact, Prochel (2006) suggested that it may be related to stabilization of the anterior body axis in the relatively altricial neonates of *T. europaea*. However, more data are needed to establish whether ossification patterns in Eulipotyphla and marsupials correspond to similar perinatal requirements; it is, however, notable that similar heterochrony is not observed in the altricial muroid rodents sampled here.

Chiroptera, represented here by members of two distant clades, are the most postcranially derived mammals in the sample due to their adaptation to active flight. It has been shown that their characteristically elongated manual digits are the result of a change in morphogen expression patterns (Sears et al. 2006). However, neither derived anatomy nor local developmental patterning result in overall similarities of postcranial ontogeny; in fact, the results demonstrate that ossification sequences within bats have diverged extensively.

With the help of increased species sampling for marsupials, it was possible to suggest apomorphic shifts for a range of marsupial clades. It is notable that out of the five marsupial clades that could be assessed in this study, four were distinguished by at least one sequence shift; in contrast, out of the ten placental clades evaluated, only five showed apomorphic sequence shifts. However, whether this can be translated into greater incidence of sequence shifts in marsupials as a whole requires more data for testing.

EVENT SIMULTANEITY

The issue of how to treat events scored as simultaneous has been discussed frequently (e.g., Velhagen 1997; Nunn and Smith 1998; Bininda-Emonds et al. 2002; Schulmeister and Wheeler 2004) and reflects the difficulties of turning continuous timing differences into categorical data. Event simultaneity is generally ascribed to a lack of resolution because it is considered unlikely that two events occur at exactly the same time. However, perceptions of the impact of event simultaneity vary. Velhagen (1997) cautioned against including simultaneous events in event-pair analysis, arguing that these are nearly always artifactual. However, most event-pair analyses have included simultaneous event scores, often with a word of caution (Smith 1997; Nunn and Smith 1998; Sánchez-Villagra 2002; Jeffery et al. 2005).

The size of the dataset analyzed here allows for a cautious assessment of the impact of including event simultaneity. Shifts toward or away from simultaneity comprise the majority of apomorphies for marsupials reported in the PAUP* analysis and Parsimov results. These event pairs distinguish virtually all placentals from all marsupials, suggesting real changes in the timing difference in these event pairs. As such, the inclusion of event simultaneity as an informative character seems warranted, at least in larger clades that consistently display simultaneity. This is because given uniform sampling, there is a high probability that simultaneity represents a real pattern of closely timed occurrence if it is reported in most or all clade members (e.g., humerus/femur in all marsupials, radius/ulna in all species sampled except *Homo*). However, it seems advisable to treat simultaneity in comparisons of small numbers of species (e.g., sister species) with caution unless sampling is very dense.

Conclusions

This study provides the largest comparative dataset to date based on which hypotheses of mammalian postcranial heterochrony could be assessed, with particular focus on heterochrony between marsupials and placentals. Heterochronically delayed ossification of the hind limb and early ossification of axial and shoulder girdle elements was recorded in marsupials, contradicting previous hypotheses of heterochronic forelimb acceleration in marsupials. This heterochrony suggests that processes such as mechanic stresses or energy allocation "trade-offs" play a major role in shaping mammalian skeletal ontogeny. Similarly extensive sequence heterochrony was not found within more inclusive therian clades. Heterochrony between marsupials and placentals largely involves changes toward or a way from event simultaneity, showing that the information content of simultaneous scores can be considerable. Future research, particularly with respect to the molecular mechanisms behind the ossification heterochrony of the marsupial

postcranial skeleton described here, has the potential to further elucidate the patterns and processes leading to postcranial heterochrony at the marsupial/placental dichotomy.

ACKNOWLEDGMENTS

For specimen loans, we thank S. Ingleby (Australian museum), C. Kemper, and D. Stemmer (South Australia Museum). C. Leigh from the Anatomical Institute, Adelaide University, and the Marsupial Immunology Group, Macquarie University, generously provided specimens for destructive sampling. For advice and discussion, we thank O. Bininda-Emonds, M. Archer, and R. Beck. A. Jones provided generous help and advice with CT acquisition. We thank two anonymous reviewers, whose suggestions improved this manuscript. This work was supported by the Swiss National Fond (3100A0–116013) to MRS-V, the U.S.A. National Science Foundation grant (#OISE-0502186) to AG, an ARC Discovery Grant to SW and an IPRS grant of the University of New South Wales and a TAP Grant # 6028 by the Nanostructural Analysis Network Organisation to VW.

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Associate Editor: F. Galis

Appendix I

ACCESSION NUMBERS

Abbreviations: AUM, Australian Museum; LM, Launceston Museum; MQU, specimens provided from the Macquarie University Marsupial reproduction laboratory; SAM, South Australian Museum; WF, specimens provided by Wendy Foster, Adelaide University

Antechinus stuartii (all AUM): 1314, 31313, 31224, 31228, 31239, 31430, 31263, 31268, 31281, 31416, 31272, 31420,

31332, 31248, 31148, 31308, 31348, 31351, 31358, 31345, 31324, 31339

Cercartetus concinnus:

SAM 4046, 5665, 18019, 15982, 127450, 22776, 11640, 13012, 16275, 20687, 16938, 16040, 14411, 4067, 22912, 15907; AUM 22939, 22921, 26252, 26253, 26254, 26255, 26257, 31132, 33131,

Dasyurus viverrinus/maculatus (DV, DM respectively): AUM DV1864, WF DV7057, WF DV7072, LM DV1964–1-111, LM DV1964–1-112, DM2119, LM DV1964–1-107, DV3115, LM DV1964–1-183, DV3112, LM DV1964–1-129, DV2206, LM DV1964–1-195, DV31125, DV744, DV3764, DM31772, DV7803, DV3623

Isoodon macrourus: 26198, 32987, 37135, 37139, 37140; 10 unnumbered specimens investigated in the collection of R. T. Gemmell, Brisbane University

Petaurus breviceps/norfolcensis (AUM): 25387, 25817, 25912, 27760, 29158, 29165, 29168, 29260, 31133, 31137, 33312, 5303, 5375, 6197, 7233, 24149, 33664, 34664, 34824, 35576, 35583, 36417

Trichosurus vulpecula:

AUM 3097, 3098, 31153, 33309, 37060, 5013, 5321, 5322; 24 unnumbered specimens investigated at the collection of R. T. Gemmell, Brisbane University and the Anatomical Institute of Adelaide University

Macropus eugenii (all MQU): 6380464, 6380Y, 8515, 2044, 61FC70CT, 63BDEBOT, 637EAIT, XIAO, 61F40, 8589, 62137A

Vombatus ursinus (all LM):

1979–1-90, 1980–1-375, 1979–1-97 , 1982–1-65 , 1979–1-127, 1980–1-110, 1980–1-70, 1982–1-100, 1982–1-73

Peromyscus melanophrys: MSV personal collection, unnumbered

Rousettus amplexicaudatus: Unnumbered specimens from the Hubrecht collection.

Appendix II. Unambiguous (double-lined arrows) and ambiguous (single-lined arrows) apomorphies uniting marsupials in a parsimony analysis using accelerated transformation (ACCTRAN) optimization, with *Alligator mississippiensis* and *Lacerta vivipara* as outgroups

Ischium/Manual phal.	$0 \rightarrow 2$
Ischium/Pubis	$1 \rightarrow 0$
Metat/Caudal	$1 \rightarrow 2$
Sternum/Metat	$2 \rightarrow 1$
Pedal phal./Manual phal.	$1 \Rightarrow 2$
Pedal phal./Sternum	$2 \rightarrow 1$
Ilium/Sacral v.	$0 \Rightarrow 1$
Ilium/Manual phal.	$0 \Rightarrow 1$
Thoracic v./Ilium	$1 \Rightarrow 0$
Ribs/Scapula	$0 \rightarrow 1$
Ribs/Thorac. v.	$0 \rightarrow 1$
Ribs/Cervic. v.	$0 \Rightarrow 1$
Metacarp./Sacral v.	$1 \rightarrow 0$
Fibula/Sacral v.	$0 \Rightarrow 1$
Fibula/Manual phal.	$0 \Rightarrow 1$
Fibula/Scapula	$1 \Rightarrow 2$
Fibula/Lumbar	$0 \Rightarrow 2$
Fibula/Thorac. v.	$1 \Rightarrow 2$
Fibula/Cervic. v.	$1 \Rightarrow 2$
Tibia/Sacral v.	$0 \Rightarrow 1$
Tibia/Manual phal.	$0 \Rightarrow 1$
Tibia/Scapula	$1 \Rightarrow 2$
Tibia/Lumbar	$0 \Rightarrow 2$
Tibia/Thorac.	$1 \Rightarrow 2$
Tibia/Cervic. v.	$1 \Rightarrow 2$
Femur/Sacral v.	$0 \Rightarrow 1$
Femur/Manual phal.	$0 \Rightarrow 1$
Femur/Scapula	$1 \Rightarrow 2$
Femur/Lumbar	$0 \Rightarrow 2$
Femur/Thorac. v.	$1 \Rightarrow 2$
Femur/Cervic. v.	$1 \Rightarrow 2$