Developmental constraint on the evolution of marsupial forelimb morphology

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Abstract. Compared with the placental mammals, marsupials are born at an almost embryonic stage, but nearly all of these neonates immediately climb or crawl to one of their mother's teats using precociously developed forelimbs. Marsupial adults also exhibit limited forelimb shape diversity relative to the members of their sister group. That the functional requirements of this natal climb have imposed a developmental constraint on marsupial forelimb evolution represents a compelling and widely accepted hypothesis, yet its resulting predictions for the comparative patterns of mammal limb shape diversity have never been tested. In order to perform such tests we conducted extensive taxonomic sampling of mammal limb morphology (including fossil specimens), and then examined these data using morphometric methods, non-parametric analyses of anatomical disparity, and phylogenetic comparative analyses of evolutionary rates. Our results strongly support the constraint hypothesis, and indicate that the highly significant differences between marsupial and placental forelimb shape diversity has been strongly influenced by different rates of morphological evolution among the distal forelimb elements in these two important mammal lineages.

Additional keywords: comparative method, developmental constraint, eutheria, forelimb, hindlimb, marsupial, metatheria, morphological evolution, morphometrics, placental mammal.

Introduction

Traits that act as key innovations or evolutionary constraints significantly affect the diversification of those organisms that posses them, but detecting the presence of such phenomena requires an ability to determine whether phylogenetic distributions of characters are distinct from random configurations. By combining extensive taxonomic sampling with appropriate comparative methods, it is possible to test for associations between specific characters and particular evolutionary patterns, and the implementation of such techniques has generated valuable information about how specific traits have directed the evolutionary trajectories of a diverse array of organisms (e.g. Bond and Opell 1998; Sears 2004; Anker *et al.* 2006; Mabuchi *et al.* 2007; Givnish *et al.* 2008; Konow *et al.* 2008). We employ such methods here in order to test anatomical predictions that follow from arguments for the presence of a developmental constraint on marsupial forelimb evolution. Such a constraint has the potential to explain a prominent trend in the morphological diversification of a major branch of the mammal radiation, and this study represents the first quantitative examination that has sought to determine whether patterns of mammal limb variation, and rates of mammal limb evolution, conform to the predictions of this hypothesis.

Mammals have undergone a massive diversification in body form since their initial divergence over 160 million years ago (Alroy 1999; Bininda-Emonds *et al.* 2007), and its members now include species that are aerial, arboreal, highly cursorial, completely fossorial, and obligately aquatic, and which range in adult size from 2 g (Kitti's hog-nosed bat) to 150 Mg (blue whale; Oldham *et al.* 2000). Part of this successful expansion into such a wide diversity of ecological niches can be attributed to the evolution of mammalian skeletal morphology, but this diversification has been far more pronounced for the placental mammals (eutherians) than for their marsupial relatives (metatherians; Lillegraven 1975; Springer 1997; Sears 2004; Polly 2007). Although there are multiple examples of marsupials that are convergent in shape and ecology with placental mammals (e.g. marsupial 'wolves', 'moles', 'mice', 'lions', and even a 'rhino'), marsupials are also notable for the absence of certain morphologies (e.g. hooves, flippers, wings) that are associated with some of the most successful clades within their sister group, Eutheria (Fig. 1; Lillegraven 1975; Polly 2007).

Some of the most striking synapomorphies of the marsupials involve their reproduction and development, including birth at an almost embryonic developmental stage (Sharman 1970; Lee and Cockburn 1985; Hughes and Hall 1988; Tyndale-Biscoe 2005). Marsupial neonates weigh less than 1 g, and organs such as the heart, brain, eyes, lungs and kidneys are still in early stages of development compared with both newborn eutherians and adult metatherians (Lee and Cockburn 1985; Cockburn 1989; Janssens
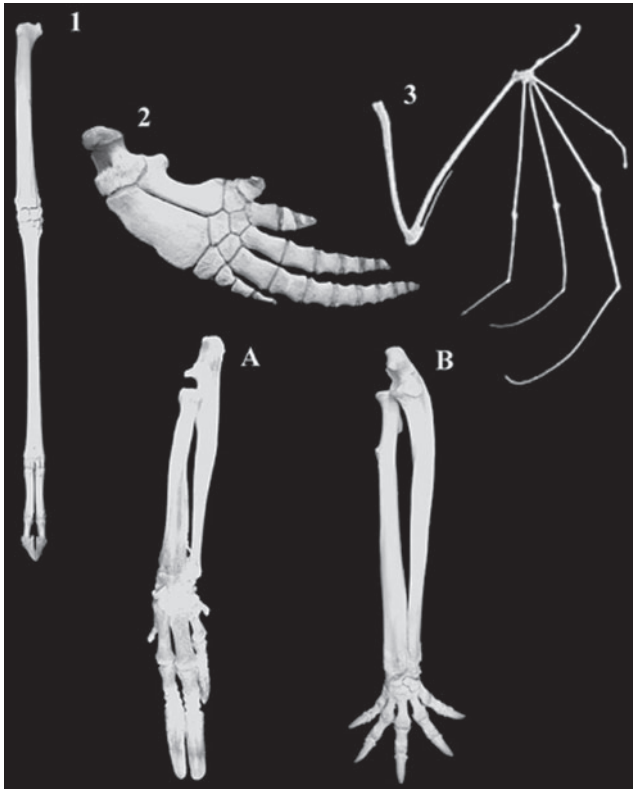
Fig. 1. Variation in mammal forelimb shape. (*1–3*) forelimbs of a dibatag (a gazelle-like antelope), dolphin and bat, placental mammals that have extremely derived limbs. (*A*) The forelimb of a spiny bandicoot, which has one of the most derived marsupial forelimb shapes. (*B*) The forelimb of a tree kangaroo, which has a typical marsupial forelimb shape.

et al. 1997; Smith 1997). Perhaps the strangest aspect of marsupial parturition is that most newborns must crawl and/or climb unaided to reach a teat where they can attach and begin to nurse, a behaviour that appears to be a synapomorphy of the lineage (Sharman 1970; Lillegraven 1975; Smith 2001; Sears 2004). The peculiarity of marsupial neonatal locomotion is increased by the fact that this crawling and climbing is achieved with large forelimbs that are highly developed in comparison to most of the remainder to the animal, including the hindlimbs (Hughes and Hall 1988; Smith 2001; Martin and Mackay 2003; Lentle *et al*. 2006).

There are some, apparently derived, alternatives to the characteristic neonatal climb among marsupials with backward-facing or poorly developed pouches, and in some of these species the mother will elevate the birth canal above the teats during parturition (Lyne 1974; Cockburn 1989; Smith 2001; Gemmell *et al*. 2002; Nelson and Gemmell 2003). These species include the bandicoots (Peramelemorphia; Lyne 1974; Smith 2001; Gemmell *et al*. 2002) and at least some of the dasyurids (Dasyuridae; Cockburn 1989; Nelson and Gemmell 2003), but in the case of the dasyurids, the forelimbs appear to retain an important locomotory function at birth (Nelson and Gemmell 2003). Although derived birth strategies exist, obligate neonatal crawling/climbing appears to be the norm among marsupials, and this behaviour is almost certainly a synapomorphy for the lineage.

There are two non-conflicting hypotheses that may account for the precocial birth of marsupials, and both concern fundamental anatomical and developmental differences between marsupial and placental mammals. The ureters lay between two lateral vaginas in marsupials, as opposed to lying lateral to the reproductive tract as in placental mammals (Sharman 1970; Springer 1997; Tyndale-Biscoe 2005), and it has been suggested that this may constitute a physical barrier that prevents marsupials from developing longer and to a larger size *in utero* (Sharman 1970). The structure of the placentae of marsupials is also fundamentally different from those of placental mammals, such that it is less likely to provide an embryo with a comparable level of protection from the mother's immune system, and this scenario may contribute to selective pressures that favour early marsupial births (Lillegraven 1975; Hughes and Hall 1988; Renfree 1993; Vaughan *et al*. 1999; Tyndale-Biscoe 2005). The term *placental mammal* does not indicate that marsupials develop without the protection and assistance of a placenta, but that eutherians possess a placental structure that is more highly developed by comparison.

Whatever the cause or causes of the precocial birth of marsupial newborns, at parturition only the forelimbs and structures associated with suckling (i.e. the jaws and tongue) are well developed, while the hindlimbs are as yet no more than limb buds (Hughes and Hall 1988; Pflieger *et al*. 1996; Smith 2001; Jeffery *et al*. 2002; Bininda-Emonds *et al*. 2003; Martin and Mackay 2003). These relatively robust forelimbs are immediately employed in locomotion by most marsupials, with the known exception of the bandicoots (Lyne 1974; Smith 2001; Gemmell *et al*. 2002). Over 30 years ago Lillegraven (1975) hypothesised that the necessity of possessing forelimbs that are well suited for climbing at such an early developmental stage limits the ability of marsupials to respond to selective pressures that would favour more derived forelimb shapes in the adults. This hypothesis has been widely cited (e.g. Klima 1987; Sanchez-Villagra and Maier 2003; Sears 2004; Croft 2006; Weisbecker and Warton 2006; Weisbecker *et al*. 2008), and Sears (2004) performed an elegant study of mammal shoulder girdle evolution and development that supports its validity, but to date there has been no quantitative, comparative examination of limb shape evolution among the therian mammals.

Since this concept has the potential to explain a major evolutionary trend, it would be unfortunate if it were to remain untested. The necessity for doing so is heightened by the fact that the number of living placental mammal species is more than 16 times greater than the number of extant marsupials (Lillegraven 1975; Kirsch 1977; Wilson and Reeder 2005), so the presence of an evolutionary constraint is not necessary in order to account for major differences in morphological diversity between these groups. In the absence of additional information, this simpler explanation would be preferable to the more complex constraint theory, but the plausibility of a connection between the unusual birth strategy of marsupials and their forelimb evolution is more than high enough to warrant investigation.

Because the placental mammals are the sister group to the marsupials (Lillegraven 1975; Graves and Westerman 2002; Bininda-Emonds *et al*. 2007) and since their neonates neither possess forelimbs that are precocialy developed relative to their caudal anatomy (Smith 2001; Martin and Mackay 2003), nor

frequently perform a natal climb, the placental mammals provide a natural control group to which marsupials may be compared (Brooks and McLennan 1993). As with all sister group studies, however, the lack of replication of either of these 'evolutionary treatments' requires that the comparison involve a high degree of analytical rigor. It is therefore fortunate that Lillegraven's hypothesis leads to multiple predictions, and that evidence in support of each would be a necessary requirement for refusing to reject his model. If the hypothesis that the morphological requirements for performing neonatal climbing has constrained marsupial forelimb evolution is correct, then a necessary corollary is that marsupial hindlimbs, which are not used or well developed at birth, should not be subject to the same level of constraint. Indirect effects should be expected, however, since most mammals are quadrupedal, and the evolution of their fore- and hindlimb morphology must therefore be correlated to some degree in many lineages. The second prediction that arises from Lillegraven's constraint hypothesis is therefore that marsupial hindlimbs should exhibit greater shape diversity than marsupial forelimbs, while the degree of morphological variation presented by placental fore- and hindlimbs should be similar.

We would expect that an evolutionarily constrained lineage might produce a lower level of species diversity in comparison to an unconstrained sister group, but if our interest lies in determining how a constraint has affected the differentiation of a second character (e.g. limb shape diversity), then disproportionate species diversity simultaneously reduces our ability to support the existence of a constraint if character diversity is the only factor being examined. In comparison to species-rich lineages, less diverse ones will tend to exhibit lower levels of character variation, whether or not a constraint exists. When investigating situations such as these, it is therefore useful to perform comparative phylogenetic tests for differential rates of character evolution. Unless it is a 'universal constraint' that is imposed by the laws of physics (*sensu* Smith *et al*. 1985), most evolutionary constraints need not function as absolute barriers to certain types of diversification, but may be more likely to operate as restrictions on rates of evolution, and it is possible to test for the presence of significantly different rates of character evolution given the existence of reliable phylogenies for the groups being examined (O'Meara *et al*. 2006).

We sought to rigorously test whether patterns of mammal limb diversification conform to the predictions generated by Lillegraven's hypothesis. In order to accomplish this, we performed extensive taxonomic sampling of therian limb shapes, and compared patterns of morphological variation among both the fore- and hindlimbs of marsupials and placental mammals. A permutation technique was then used to test for statistically significant differences in morphological disparity between different mammal limb groups (e.g. marsupial forelimbs versus placental forelimbs, marsupial forelimbs versus marsupial hindlimbs, etc.). We also utilised published phylogenetic information to construct composite trees for the taxa we examined, and we assigned divergence times to the nodes of these trees based on the available literature. This information was used in conjunction with our anatomical data in order to perform comparative phylogenetic tests of the prediction that marsupials

and placental mammals have experienced dissimilar rates of limb-shape evolution.

Materials and methods

Specimens

Specimens from 94 species that represent nearly all extant marsupial families (19 of 21), slightly more than half of the extant placental mammal families (59 of 114), and multiple extinct species were examined. An effort was made to include the extremes of mammal limb-shape diversity within our data. We examined select fossil specimens in order to include unique limb morphologies not present among extant species. This was particularly important in the case of the marsupials, since several species that possessed divergent limb morphologies are now extinct (Wroe and Milne 2007; Weisbecker and Archer 2008). See Appendices 1 and 2 for specimen lists. Taxonomic classifications are after Wilson and Reeder (1993).

Osteological measurements

The morphometric analysis of structures, such as limbs, that have multiple moveable elements requires the use of measurement-based techniques. The limbs of museum specimens are preserved in a wide range of configurations, and many of them cannot be reconfigured (e.g. bent at the joints), so that all of the limbs have their bones in homologous positions when coordinate data for anatomical landmarks are collected (at least not without breaking or damaging the specimens). Coordinate-based methods (geometric morphometrics) would therefore introduce error via positional differences among the various limb elements were they to be used to describe and quantify variation in whole-limb morphology. This problem is exacerbated by the fact that the great diversity of mammal limb shapes does not permit the homologous positioning of all limb elements from every species even if the specimens are completely flexible. The high degree of limb shape evolution being examined also introduces certain problems with homology, since the same measurements must be taken from each specimen, and since numerous features of these limb skeletons have been lost, fused or greatly altered. In order to retain measurement homology among the limbs of such diverse animals as bats, whales, kangaroos and horses, the measurements must remain relatively simple, but in order to adequately capture sufficient shape information for our testing purposes, the number of the measurements made must, in turn, be reasonably large. We recorded 50 measurements for each limb, measuring one fore- and one hindlimb from each individual. Most measurements were taken from the manus and pes of the limb since most of the limb bones are present within these regions. Single specimens of either sex were selected to represent each species, and adult specimens were used in all cases except for *Elephas maximus* and *Lycopsis longirostris*. See Appendix 3 for the limb measurements used.

Since it was necessary to record homologous measurements from all specimens, measurements were made of those bones thought to have been present in the ancestor to both marsupials and placental mammals. These include five digits on both the manus and pes, with two phalanges in the hallux and pollex, and three phalanges in the remaining digits (Romer 1966; Hamrick 2001; Richardson and Chipman 2003). Measurements of bones

that have been lost in some species were recorded as zero. In cases of hyperphalangy (i.e. polyphalangy), where digits are composed of more than three phalanges, only data from the three most proximal phalanges were analysed. This condition is unknown in mammals outside of the Cetacea, although some pinnipeds possess phalanx-like cartilaginous extensions of their digits (Richardson and Chipman 2003). Data from those species that have lost their hindlimbs (i.e. sirenians and cetaceans) were not included in the hindlimb analyses.

For long bones of the zeugopod (i.e. the tibia, fibula, radius and ulna) that were fused with one bone tapering to a point, the length of the tapering bone was measured to that point and the width at that end was recorded as zero. The location of such points was determined by examining suture lines. If two bones were fused equally (e.g. the cannon bones of some hoofed mammals), bone widths were measured from the suture line outward. Most measurements were taken using a set of Fowler/Sylvac Ultra-cal III digital calipers (Fred V. Fowler Co. Inc., Newton, MA) interfaced with a computer running the CalExcel software program by Heaton (available from the author at http://www.usd.edu/esci/programs/). The larger measurements were taken using a tape measure when necessary. A dissecting light microscope was used when examining the bones of smaller species. All measurements were recorded to the nearest 0.1 mm when using calipers, and to the nearest 0.5 mm when using a tape measure.

Shape analyses

Morphometric analyses aimed at describing shape diversity require transformation of the raw data in order to reduce the effect of size differences on shape descriptions. Our first step towards performing such a transformation was to natural log–transform the data, but since measurements of zero cannot be log-transformed (e.g. for missing structures), all measurements were first translated by adding 0.1 mm. This transformation should not distort the results of our analyses.

After this initial transformation, separate principal components analyses of the fore- and hindlimb datasets were performed. The second step for the reduction of size effects was then implemented using Burnaby's method, which assumes that the first principal component (PC) of a principal component analysis (PCA) is strongly correlated with size, and that the removal of this component ('shearing' the data) will reduce the effects of size on descriptions of shape (Burnaby 1966; Rohlf and Bookstein 1987; Warheit et al. 1999). Subsequent to this final transformation, PC score plots of fore- and hindlimb shape were used to describe the patterns and extent of mammal limb diversity, and the loadings of each measurement variable on the individual PC axes were used to determine the type of shape variation described by each axis. We chose to report results for the first two PC shape axes that, when taken together, accounted for more than 70% of the total shape variation in each of the two datasets. The software application NTSYS (Exeter Software, Setauket, NY) was used to perform shearing and PCA.

Shape diversity calculations and comparisons

Shape diversity (i.e. morphological disparity) was measured as the total variance of each dataset (Foote 1997; Warheit et al.

1999), and a custom permutation program (LaBarbera 2002) written using the software program HiQ (National Instruments Corp., Austin, TX), was used to determine whether the shape diversity values calculated for individual limb groups were significantly different. This program first calculates the difference in variance between two matrices of limb data. The two datasets are then pooled into one matrix, and two replicate matrices equal in size to the original matrices being compared are created by random allocation of rows (specimens), without replacement. The difference between the variance values of these generated matrices are then calculated. This procedure was repeated for 10 000 iterations for each test in order to generate a distribution of differences. The percentile location of the original difference in shape diversity on the generated distribution determines the *P*-value of the result.

Comparative phylogenetic tests for equal rates of morphological evolution

The software program Mesquite (Maddison and Maddison 2008) was used to construct a phylogeny for the therian mammal species examined in this study (see Figs 2, 3 for the separate metatherian and eutherian portions of this tree). Tree topology and estimated divergence dates were determined from published accounts of mammal relationships, palaeontology and taxonomy (Romer 1966; de Muizon 1999; Nowak 1999; Krajewski et al. 2000; Murphy et al. 2001, 2007; Delsuc et al. 2002; Waddell and Shelley 2003; Asher et al. 2004; Gaudin 2004; Reyes et al. 2004; Fernandez and Vrba 2005; Jansa and Voss 2005; Wilson and Reeder 2005; Fulton and Strobeck 2006; Yu and Zhang 2006; Bininda-Emonds et al. 2007; Pujos and De Iuliis 2007; Rohland et al. 2007; Beck 2008; Kullberg et al. 2008; Meredith et al. 2008). The divergence date for the metatheria and eutheria was set at 147.7 million years ago (Bininda-Emonds et al. 2007). Branch lengths were set proportional to time. Terminal branches leading to extinct species were shortened to the age of last appearance, except for extinctions more recent than 100 000 years BP, where branches were extended to the recent.

The phylogeny with branch-length information was used in conjunction with morphological data in order to perform parametric bootstrapping tests for similar rates of character evolution using the computer program Brownie (see O'Meara et al. 2006 for details). Tests for similar rates of change between marsupials and placental mammals were performed for two types of characters: the quantity of certain groups of distal limb elements (digits on the manus, digits on the pes, phalangeal bones in the manus, phalangeal bones in the pes, metacarpals, and metatarsals); and changes in certain limb proportions (the fraction of total limb length composed of the stylopod, zeugopod, autopod, wrist or ankle, manus or pes, and the longest digit on either the manus or pes). Principal component scores were not used as characters in these phylogenetic comparative tests due to the fact that they lack biological significance when treated as single univariate characters (Rohlf 1998; Zelditch et al. 1998; Monteiro 2000). The number of bootstrapping runs was set to 1000 for each test.
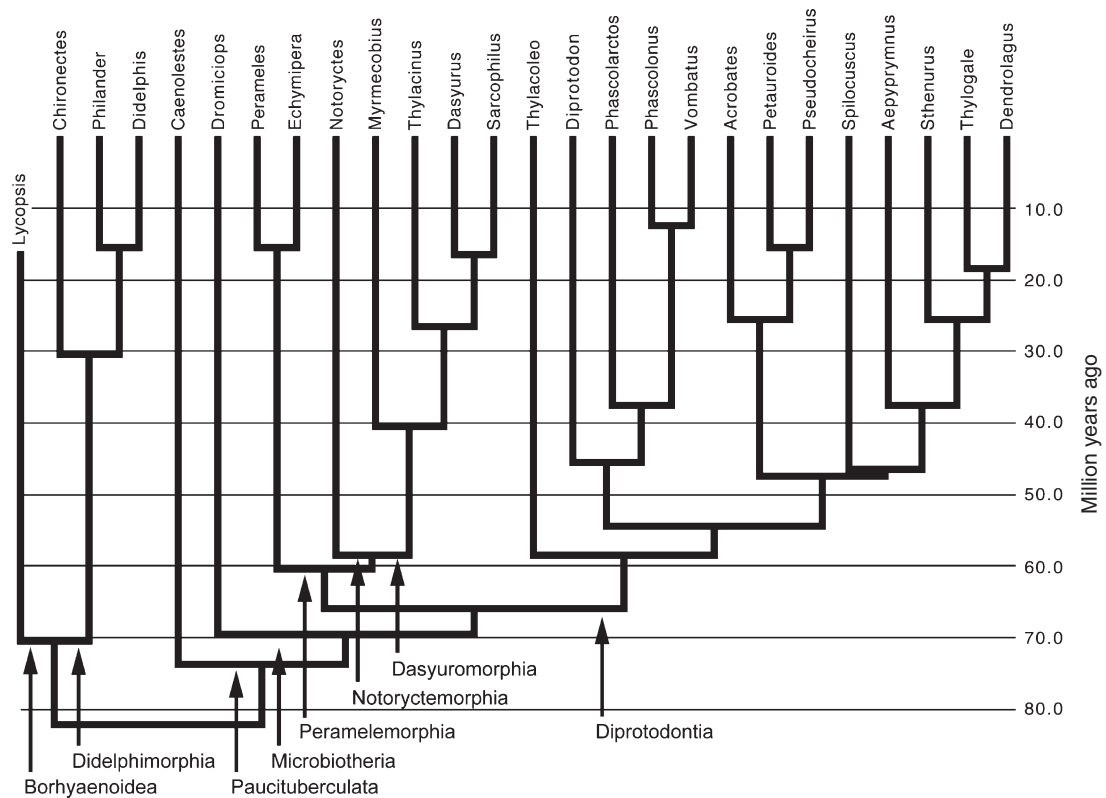
Fig. 2. Composite phylogeny of the marsupial species examined in this study with estimated divergence dates. The terminal branch for species that became extinct less than 100 000 years ago extend to the present. Taxonomic orders are labelled.

Results

Marsupials occupy a subset of the forelimb shape space inhabited by placental mammals (Figs 4, 5) and most of the placental forelimbs that plot outside of marsupial forelimb space belong to functional groups with no marsupial analogues. Marsupial hindlimbs also occupy most of the placental region of hindlimb shape space (Figs 4, 6). Only 20 placental species (29.85% of those analysed) plot outside of the marsupial hindlimb polygon as compared with 36 (53.73%) of the placental species in the forelimb shape space (Figs 4–6). Functional groupings among placental mammals are also more distinct for the forelimbs than for the hindlimbs (Fig. 4). Of the placental hindlimbs that lie outside the marsupial polygon, approximately half are similar in shape to many marsupial hindlimbs (Figs 4, 6). The extinct, single-toed kangaroo (*Sthenurus occidentalis*) occupies a small region of exclusively marsupial limb space (Figs 4, 6). This species exhibits a distinct pattern of marsupial digit reduction where the 4th digit is dominant as opposed to the 3rd, as is usual in placental mammals (Marshall 1974; Wells and Tedford 1995).

The difference in forelimb shape diversity between marsupials and placentals is highly significant ($P = 0.004$), but marsupial hindlimbs are not significantly less diverse than placental hindlimbs ($P = 0.13$; Table 1). Marsupial hindlimbs also exhibit more than 54% greater shape diversity than do the forelimbs of the same specimens (a non-significant difference, $P = 0.095$), while placental mammal fore- and hindlimbs display very similar levels of morphological diversity ($P = 0.484$; Table 1). There were also significant evolutionary rate differences between marsupials and placental mammals in regard to changes in the number of distal limb elements present in the forelimb (i.e. the numbers of digits, phalanges, and metacarpals), but this was not true for the hindlimb (Table 2). Rates of evolutionary change in limb proportions did not differ between the two lineages (Table 2).

Discussion

The results of quantitative comparisons of limb shape diversity (Table 1), phylogenetic tests for differences in rates of evolutionary change in limb morphology (Table 2), and the comparative patterns of limb shape variation (Figs 4–6), consistently support the predictions of Lillegraven's constraint hypothesis. The results also indicate that there are patterns to the types of limb functions that have not evolved among the marsupials. An important portion of these are associated with quadrupedal walking and/or running with the limbs oriented directly below the body (as opposed to the more sprawling posture associated with climbing). The artiodactyls, perissodactyls, and many cavioid rodents, in particular, have several adaptations for cursoriality not found among the marsupials. Their locomotion involves the coordination of similar, highly derived fore- and hindlimbs that have reduced distal elements.

Interestingly, the highly aquatic placental mammals whose forelimbs have evolved into flippers, a structure that has arisen
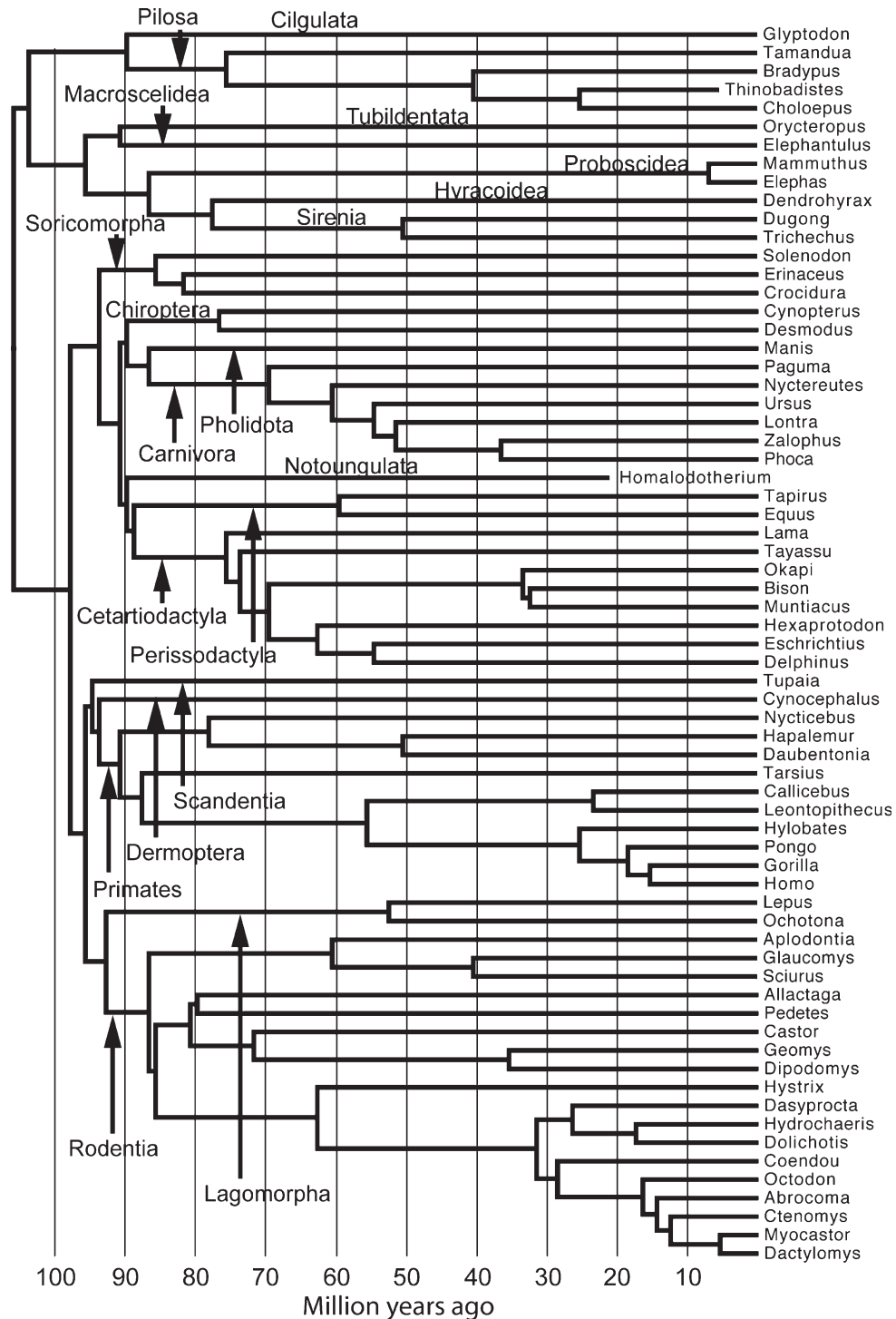
Fig. 3. Composite phylogeny of the placental mammal species examined in this study with estimated divergence dates for nodes. Although the placement of the Cetacea renders the order Artiodactyla paraphyletic, this has no effect on our analyses. Taxonomic orders are labelled.

independently three times in this group (sirenians, pinnipeds, and cetaceans) do not form a distinct cluster, and two of the species analysed have forelimbs that lie within the marsupial region (Figs 4, 5). For sirenians and cetaceans this may be due to the fact

that the distal bones of their limbs function together as a single, rigid element. These bones are uniformly flattened and closely articulated with each other, but because the limb operates as a single unit, the shapes of the individual bones within the flipper
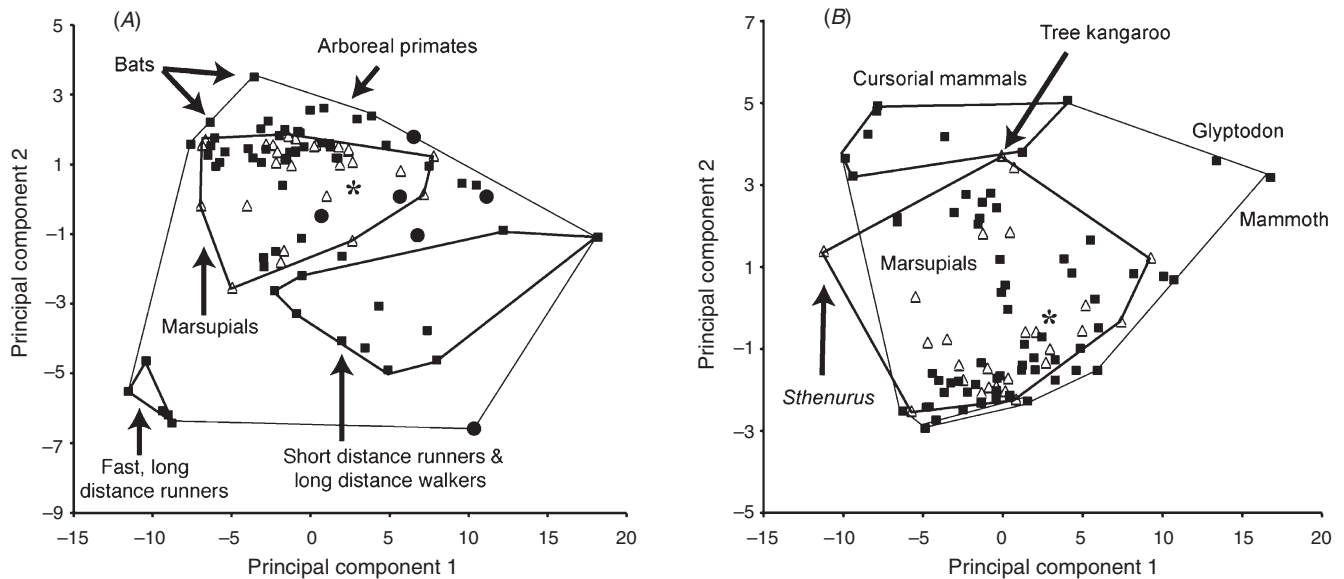
Fig. 4. Distribution of mammal limb shapes. △ = marsupials; ■ = placentals; ✱ = echidna ● = placentals with flippers. (*A*) Forelimbs. Principal component 1 describes 65.46% of the variation in the forelimb data and is positively correlated with limb shapes that have proximal limb elements that are robust relative to distal limb elements. Principal component 2 (12.67%) is positively correlated with manus shapes that have retained the 5th digit and that have uniformly well developed bones in all digits. (*B*) Hindlimbs. Principal component 1 describes 60.24% of the variation in the hindlimb data and is positively correlated with limb shapes that have proximal limb elements that are robust relative to distal limb elements. Principal component 2 (11.60%) is positively correlated with limb shapes that have well developed 3rd digits and reduced or absent 5th digits. For identification of the species associated with each point see Figs 5 and 6.

may be of reduced importance and therefore free to evolve in different ways.

No marsupials have evolved flippers, and the only semiaquatic species, the yapok (*Chironectes minimus*), swims exclusively with its hindlimbs (Nowak 1999). Apart from the putative evolutionary constraint imposed by the morphological requirements for climbing at birth, the precocial nature of marsupial parturition makes the evolution of a fully aquatic marsupial problematic. The inclusion of data from obligately aquatic placental species (i.e. *Eschrichtius robustus*, *Delphinus delphis*, *Dugong dugon*, and *Trichechus manatus*), while making an important contribution to the comprehensive nature of the study, may therefore represent an evolutionary comparison that has little to do with Lillegraven's hypothesis. However, of these four species, only the grey whale (*E. robustus*) showed any appreciable differentiation of forelimb shape relative to the marsupials as a group, and all of them retained the ancestral mammalian number of limb bones (apart from extra, distal phalanges in the cetaceans, which were excluded from the dataset), so the inclusion of these species in the data should not affect our conclusions.

It is notable that the hindlimbs of the tree kangaroo (*Dendrolagus lumhotzi*) are very similar to those of the more cursorial placental animals. Although this species is largely arboreal, its hindlimb morphology is similar to that of most other extant kangaroos, a lineage of efficient saltators. Most kangaroo hind feet have greatly enlarged 3rd and 4th digits (or just an enlarged 4th digit, as in *Sthenurus* and some other extinct kangaroos) with the other digits strongly reduced or absent, while their forelimbs largely conform to the ancestral therian

morphology (Fig. 1). The kangaroos present an obvious example of species whose anterior and posterior limb shapes are very different, and the forelimbs of terrestrial kangaroos are not used at all when bounding at higher speeds, the gait which is their most energetically efficient (Windsor and Dagg 1971; Baudinette *et al.* 1987; Bennett 2000). It is informative that the most efficient mode of locomotion for those marsupials with the most highly derived hindlimbs involves functionally decoupling the anterior and posterior limbs.

If the constraint hypothesis is valid, then marsupial forelimbs should be able to diversify if the constraint is removed (or diversify at a faster rate). Alternative, and presumably derived, marsupial birth processes have been described in bandicoots and dasyurids, where the mothers elevate their urogenital sinuses during birth such that gravity aids their young in moving towards the teats (Lyne 1974; Cockburn 1989; Gemmell *et al.* 2002; Nelson and Gemmell 2003). In dasyurids the forelimbs still appear to play an active part in neonatal locomotion (Nelson and Gemmell 2003), and functional requirements for newborns' forelimbs may still exert some level of constraint on the evolution of adult dasyurid forelimb shapes.

The bandicoots, however, have a much more highly derived birth process, in which the newborns sometimes remain attached to their mother for some time by means of a placental stalk, and where they wriggle downwards and into the posteriorly facing pouch without employing their forelimbs (Lyne 1974; Cockburn 1989). These species also possess some of the most derived marsupial forelimbs (Fig. 1). Along with the two species of marsupial moles (Notoryctidae, *Notoryctes*), about which extremely little is known, the bandicoots are the only other
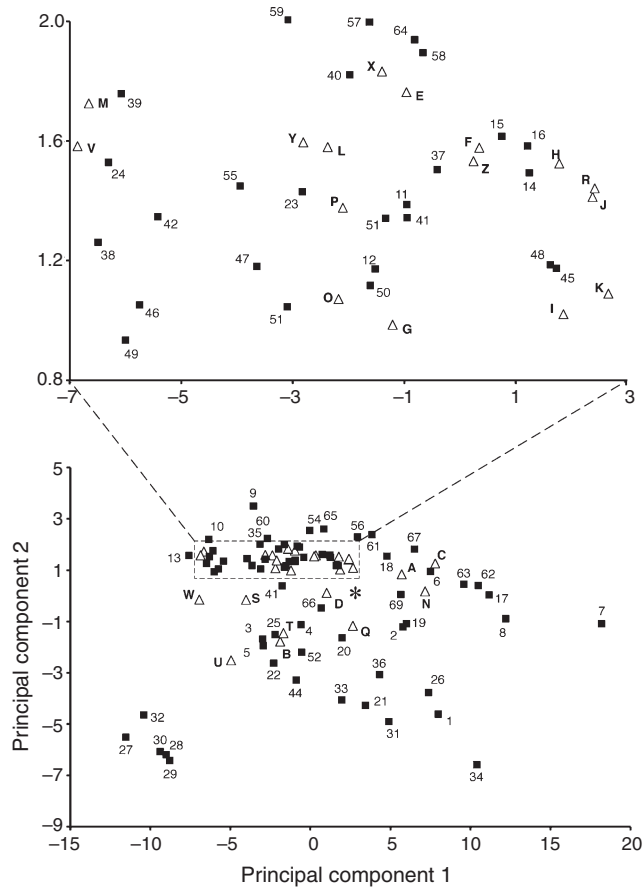
Fig. 5. Labelled distribution of mammal forelimb shapes. △ = marsupials; ■ = placentals; ✳ = echidna. Axes and coordinates of individual species are identical to those in Fig. 4*A*. Letters and numbers identifying species are the same as those listed in Appendix 1.

Fig. 6. Labelled distribution of mammal hindlimb shapes. △ = marsupials; ■ = placentals; ✳ = echidna. Axes and coordinates of individual species are identical to those in Fig. 4*B*. Letters and numbers identifying species are the same as those listed in Appendix 1.

marsupials to have lost digits on the manus (Szalay 1994; Nowak 1999; Withers *et al*. 2000). This condition occurs in several peramelid species, and the recently extinct pig-footed bandicoot (*Chaeropus ecaudatus*) had only two functional digits on the forelimb and one on the hindlimb (Nowak 1999). The only marsupials that are known to not use their forelimbs immediately after birth therefore possesses some of the most highly derived marsupial forelimb shapes.

The corollary to Lillegraven's hypothesis is also supported by our data. The hindlimbs of marsupials are not significantly constrained relative to placental mammals (Tables 1, 2), although they do occupy less of the available morphospace (Table 1; Figs 4, 6). This lower level of shape variation might be due to developmental correlations with the forelimbs, or functional
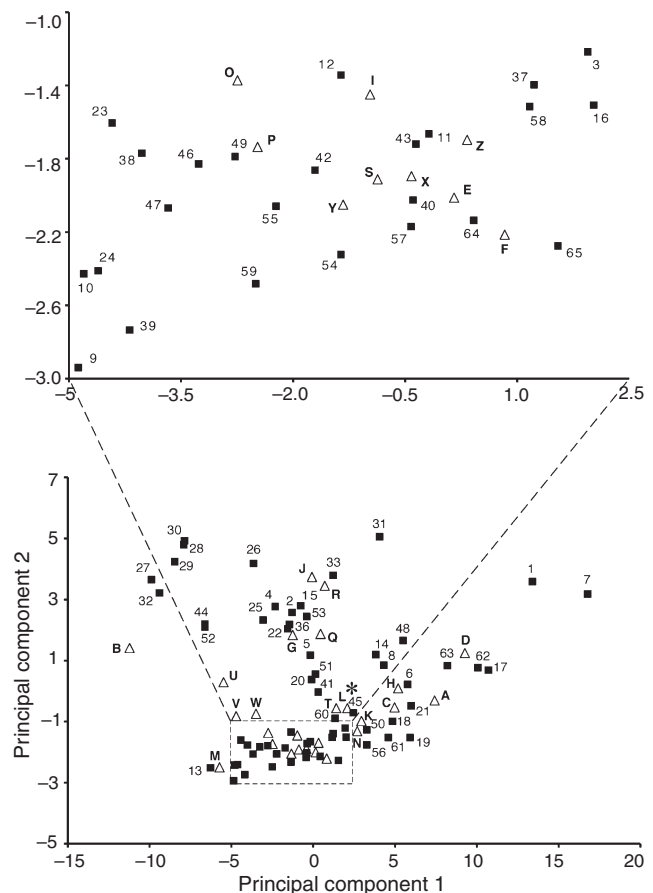
correlations between the anterior and posterior limbs that are due to either locomotory requirements or other aspects of marsupial ecology.

Sears (2004) performed a comparative study of the morphology and development of the shoulder girdles in marsupial and placental mammals, and provided strong evidence that the functional requirements of the natal climb had constrained the evolution of this structure. The shoulder girdle anchors the muscles that provide the force exerted during this climb (Sears 2004), and the constraints examined by Sears are therefore directly associated with propulsion. The other major physical requirement for climbing is purchase; the newborn marsupial must also be able to grip its mother's fur well enough to apply climbing forces with its forelimbs.

Table 1. Estimates of morphological diversity and results of permutation tests for equal levels of shape disparity

Morphological group	Shape disparity	Shape disparity comparison	P
Marsupial forelimbs	46.066	Marsupial forelimbs *v.* placental forelimbs	0.004*
Marsupial hindlimbs	71.151	Marsupial hindlimbs *v.* placental hindlimbs	0.136
Placental forelimbs	109.893	Marsupial forelimbs *v.* marsupial hindlimbs	0.095
Placental hindlimbs	100.947	Placental forelimbs *v.* placental hindlimbs	0.484

Table 2.   Results of phylogenetic tests for similar rates of limb character evolution
FL = forelimb length, HL = hindlimb length. Characters with significantly different rates are shown in bold

	ln(L) of a single evolutionary rate for marsupials and placentals	ln(L) of separate evolutionary rates for marsupials and placentals	Bootstrapping P
Forelimb character (manus)			
Digit number	65.60	12.23	0.022
Phalangeal number	195.52	177.99	0.001
Metacarpal number	68.42	−790.71	<0.001
Stylopod length/FL	−166.46	−166.50	0.873
Zeugopod length/FL	−140.22	−140.49	0.672
Autopod length/FL	−142.91	−144.39	0.294
Wrist length/FL	−225.95	−230.69	0.068
Manus length/FL	−136.49	−137.30	0.432
Longest digit length/FL	−167.98	−168.00	0.884
Hindlimb character (pes)			
Digit number	131.06	131.02	0.855
Phalangeal number	224.30	224.23	0.837
Metatarsal number	127.78	127.74	0.866
Stylopod length/HL	−98.66	−101.64	0.116
Zeugopod length/HL	−126.37	−129.70	0.123
Autopod length/HL	−113.38	−116.84	0.114
Ankle length/HL	−213.73	−213.79	0.842
Pes length/HL	−117.24	−120.71	0.101
Longest digit length/HL	−158.74	−161.48	0.140

The results of our tests for differences in evolutionary rates suggest that marsupials have not rapidly deviated from the ancestral morphology of the therian manus (Table 2). Most marsupials retain five digits, along with most of the phalanges and metacarpals associated with them, and this pattern is consistent with what is to be expected if they require a maximal amount of surface area at the distal ends of their forelimbs as newborns. Most marsupial neonates also have forelimb digits that end in pointed claws (Hughes and Hall 1988; Martin and Mackay 2003), which would further contribute to their ability to securely plant each manus while the opposite limb is being moved forward. The proportions of the different regions of marsupial forelimbs appear to be as free to evolve as those in the limbs of the placental mammals (Table 2), so the morphological effects of Lillegraven's constraint appear to be most highly concentrated at either end of the forelimb; most of their shoulder girdles maintain a form that is adapted for anchoring a newborn's climbing muscles, and most of their distal limb elements have been retained in order to permit the efficient transfer of this force to their mother's fur during a climb.

An unanswered question is why must newborn marsupials climb at all? Altricial newborns are far from rare among the placental mammals, and there is no reason to suppose that most marsupial mothers are incapable of manipulating their neonates. A plausible and testable hypothesis for why this behaviour might be of adaptive value would be a welcome addition to our attempts to understand why marsupial forelimb evolution has taken this curious path.

There is now a great deal of comparative evidence, both quantitative and otherwise, that indicates that Lillegraven was correct, and that his theory explains a significant portion of therian morphological evolution. The patterns of mammal limb shape diversity (both fore- and hind-), the results of both quantitative morphological analyses and phylogenetic analyses of evolutionary rates, the comparative evidence provided by the hindlimbs of kangaroos and the forelimbs of bandicoots, and the structure and development of marsupial shoulder girdles, all consistently support the concept that the need for forelimbs capable of climbing at birth has limited the morphological and functional evolution of the marsupials. This important developmental difference has had far-reaching consequences for ecological divergence, and those mammals whose newborns are not required to undergo a natal climb have been able to diversify in ways that have allowed them to be highly successful inhabitants of such diverse ecological niches as the oceans and the night-time sky.

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Appendix 1.   Taxonomic list of the species examined
An asterisk denotes fossil specimens

Metatheria
  Diprotodontia
    A *Phascolonus gigas* (giant wombat)*
    B *Sthenurus occidentalis* (a one-toed kangaroo)*
    C *Diprotodon australis* (marsupial 'rhino')*
    D *Thylacoleo carnifex* (marsupial 'lion')*
    E *Petauroides volans* (greater gliding possum)
    F *Spilocuscus maculatus* (spotted cuscus)
    G *Aepyprymnus rufescens* (rufous 'rat'-kangaroo)
    H *Phascolarctos cinereus* (koala)
    I *Thylogale billardierii* (red-bellied pademelon)
    J *Dendrolagus lumholtzi* (Lumholtz's tree kangaroo)
    K *Vombatus ursinus* (common wombat)
    L *Pseudocheirus peregrinus* (ring-tailed possum)
    M *Acrobates pygmaeus* (pygmy gliding possum)
  Borhyaenoidea
    N *Lycopsis longirostrus* (a borhyaenid)*
  Dasyuromorphia
    O *Myrmecobius fasciatus* (numbat)
    P *Dasyurus viverriunus* (quoll)
    Q *Sarcophilus laniarius* (Tasmanian devil)
    R *Thylacinus cynocephalus* (thylacine)
  Peramelemorphia
    S *Perameles gunnii* (long-nosed bandicoot)
    T *Echymipera* sp. (spiny bandicoot)
  Notoryctemorphia
    U *Notoryctes typhlops* (marsupial 'mole')
  Paucituberculata
    V *Caenolestes fuliginosus* (common 'shrew' opossum)
  Microbiotheria
    W *Dromiciops gliroides* (monito del monte)
  Didelphimorphia
    X *Chironectes minimus* (yapok)
    Y *Philander opossum* (grey 'four-eyed' possum)
    Z *Didelphis virginiana* (Virginia opossum)
Eutheria
  Cilgulata
    1 *Glyptodon* sp. (glyptodon)*
  Pilosa
    2 *Thinobadistes segnis* (giant ground sloth)*
    3 *Tamandua mexicana* (tamandua)
    4 *Bradypus variegatus* (three-toed sloth)
    5 *Choloepus didactylus* (two-toed sloth)
  Notoungulata
    6 *Homalodotherium cunninghami*
    (a notoungulate)*
  Proboscidea
    7 *Mammuthus* sp. (Mammoth)*
    8 *Elephas maximus* (Asiatic elephant)
  Chiroptera
    9 *Cynopterus brachyotis* (short-nosed fruit bat)
    10 *Desmodus rotundus* (vampire bat)
  Soricimorpha
    11 *Solenodon paradoxus* (Hispaniolan solenodon)
    12 *Erinaceus europaeus* (Eurasian hedgehog)
    13 *Crocidura* sp. (white-toothed shrew)
  Carnivora
    14 *Lontra canadensis* (river otter)
    15 *Nyctereutes procyonoides* (raccoon dog)
    16 *Paguma larvata* (masked palm civet)
    17 *Zalophus californianus* (California sea lion)
    18 *Ursus thibetanus* (Asiatic black bear)
    19 *Phoca vitulina* (harbor seal)

Pholidota
    20 *Manis javanica* (Mayalan pangolin)
Tubulidentata
    21 *Orycteropus afer* (aardvark)
Lagomorpha
    22 *Lepus capensis* (Cape hare)
    23 *Ochotona rufescens* (pika)
Macroscelidea
    24 *Elephantulus brachyrhynchus* (long-eared elephant shrew)
Hyracoidea
    25 *Dendrohyrax arboreus* (tree hyrax)
Perissodactyla
    26 *Tapirus indicus* (Asiatic tapir)
    27 *Equus caballus* (Przewalski's horse)
Cetartiodactyla
    28 *Bison bison* (bison)
    29 *Lama glama* (Llama)
    30 *Okapi johnstoni* (okapi)
    31 *Hexaprotodon liberiensis* (pygmy hippopotamus)
    32 *Muntiacus* sp. (muntjac)
    33 *Tayassu pecari* (white-lipped peccary)
    34 *Eschrichtius robustus* (grey whale)
    35 *Delphinus delphis* (common dolphin)
Rodentia
    36 *Hydrochaeris hydrochaeris* (capybara)
    37 *Coendou mexicanus* (prehensile-tailed porcupine)
    38 *Allactaga tetradactyla* (four-toed jerboa)
    39 *Glaucomys volans* (Eastern flying squirrel)
    40 *Sciurus niger* (fox squirrel)
    41 *Geomys bursarius* (Eastern American pocket gopher)
    42 *Dipodomys deserti* (desert kangaroo rat)
    43 *Aplodontia rufa* (mountain beaver)
    44 *Dolichotis patagonum* (Patagonian cavy)
    45 *Hystrix* sp. (old world porcupine)
    46 *Abrocoma cinerea* (chinchilla rat)
    47 *Ctenomys* sp. (tuco-tuco)
    48 *Castor canadensis* (beaver)
    49 *Octodon degus* (degu)
    50 *Myocastor coypus* (nutria)
    51 *Dactylomys dactylinus* (coro-coro)
    52 *Dasyprocta punctata* (agouti)
    53 *Pedetes capensis* (springhare)
Dermoptera
    54 *Cynocephalus volans* (flying lemur)
Sandentia
    55 *Tupaia glis* (tree shrew)
Primates
    56 *Hylobates syndactylus* (siamang)
    57 *Callicebus donacophilus* (titi monkey)
    58 *Nycticebus coucang* (slow loris)
    59 *Tarsius syrichta* (tarsier)
    60 *Leontopithecus rosalia* (golden lion tamarin)
    61 *Pongo pygmaeus* (orangutan)
    62 *Gorilla gorilla* (gorilla)
    63 *Homo sapiens* (human)
    64 *Hapalemur griseus* (bamboo lemur)
    65 *Daubentonia madagascariensis* (aye-aye)
Sirenia
    66 *Dugong dugong* (dugong)
    67 *Trichechus manatus* (manatee)
Prototheria
  Monotremata
    Zaglossus bruijni (echidna)

Appendix 2.   Alphabetical list of specimens with museum identification numbers

AMNH, American Museum of Natural History; FLIN, Flinders University; FLMNH, Florida Museum of Natural History; FMNH, Field Museum of Natural History; MVZ, Museum of Vertebrate Zoology, University of California, Berkeley; SAMA, South Australian Museum; USNM, United States National Museum of Natural History

Species	ID number	Museum	Species	ID number	Museum
Abrocoma cinerea	395718	USNM	*Hystrix* sp.	49348	USNM
Acrobates pygmaeus	37463	USNM	*Lama glama*	display	FLMNH
Aepyprymnus rufescens	48018	FMNH	*Leontopithecus rosalia*	148059	FMNH
Allactaga tetradactyla	84157	FMNH	*Lepus capensis*	42407	FMNH
Aplodontia rufa	271138	USNM	*Lontra canadensis*	53922	FMNH
Bison bison	15577	FMNH	*Lycopsis longirostrus*	38061	MVZ
Bradypus variegatus	111636	USNM	*Mammuthus* sp.	display	FLMNH
Caenolestes fuliginosus	18604	FMNH	*Manis javanica*	68742	FMNH
Callicebus donacophilus	121659	FMNH	*Muntiacus* sp.	244374	AMNH
Castor canadensis	21230	USNM	*Myocastor coypus*	1994	USNM
Chironectes minimus	58807	FMNH	*Myrmecobius fasciatus*	19982	FMNH
Choloepus didactylus	60058	FMNH	*Notoryctes typhlops*	49545	USNM
Coendou mexicanus	15611	FMNH	*Nyctereutes procyonoides*	59013	FMNH
Crocidura sp.	546949	USNM	*Nycticebus coucang*	108856	FMNH
Ctenomys sp.	132277	USNM	*Ochotona rufescens*	96823	FMNH
Cynocephalus volans	61032	FMNH	*Octodon degus*	397332	USNM
Cynopterus brachyotis	140647	FMNH	*Okapi johnstoni*	51902	AMNH
Dactylomys dactylinus	549596	USNM	*Orycteropus afer*	135082	FMNH
Dasyprocta punctata	261397	USNM	*Paguma larvata*	98647	FMNH
Dasyurus viverrinus	42758	FMNH	*Pedetes capensis*	49647	USNM
Daubentonia madagascariensis	199694	USNM	*Perameles gunnii*	160056	FMNH
Delphinus delphis	display	FMNH	*Petauroides volans*	60908	FMNH
Dendrohyrax arboreus	86883	FMNH	*Phascolarctos cinereus*	521411	USNM
Dendrolagus lumholtzi	65258	AMNH	*Phascolonus gigas*	display	AMNH
Desmodus rotundus	48305	FMNH	*Philander opossum*	304647	USNM
Didelphis virginiana	397200	USNM	*Phoca vitulina*	77933	AMNH
Dipodomys deserti	34917	FMNH	*Pongo pygmaeus*	53203	FMNH
Diprotodon australis	display	SAMA	*Pseudocheirus peregrinus*	221165	USNM
Dolichotis patagonum	175890	USNM	*Sarcophilus laniarius*	47166	FMNH
Dromiciops gliroides	50072	FMNH	*Sciurus niger*	47748	FMNH
Dugong dugon	display	USNM	*Solenodon paradoxus*	51068	FMNH
Echymipera sp.	60701	FMNH	*Spilocuscus maculatus*	31752	FMNH
Elephantulus brachyrhynchus	83539	FMNH	*Sthenurus occidentalis*	P20820	FLIN
Elephas maximus	34918	FMNH	*Tamandua mexicana*	18835	FMNH
Equus caballus	202718	AMNH	*Tapirus indicus*	41393	FMNH
Erinaceus europaeus	271142	USNM	*Tarsius syrichta*	142007	FMNH
Eschrichtius robustus	display	USNM	*Tayassu pecari*	49848	FMNH
Geomys bursarius	123426	FMNH	*Thinobadistes segnis*	DE 21547	FLMNH
Glaucomys volans	44885	FMNH	*Thylacinus cynocephalus*	155407	USNM
Glyptodon sp.	display	FMNH	*Thylacoleo carnifex*	display	FLIN
Gorilla gorilla	154559	USNM	*Thylogale billardierii*	153532	USNM
Hapalemur griseus	83668	USNM	*Trichechus manatus*	display	USNM
Hexaprotodon liberiensis	2423	AMNH	*Tupaia glis*	104809	FMNH
Homalodotherium cunninghami	display	FMNH	*Ursus thibetanus*	90184	AMNH
Homo sapiens	225267	USNM	*Vombatus ursinus*	22987	USNM
Hydrochaeris hydrochaeris	53846	FMNH	*Zaglossus bruijni*	22992	USNM
Hylobates syndactylus	60340	FMNH	*Zalophus californianus*	15548	FMNH

Appendix 3.   Limb measurements collected

Humerus:
  1. Length of the humerus long axis
  2. Greatest width of the head of the humerus
  3. Distance between the lateral and medial epicondyles at the distal end of the humerus
Ulna:
  1. Length of the ulna long axis, including the head
  2. Greatest width at the proximal end of the ulna
  3. Greatest width at the distal end of the ulna
Radius:
  1. Length of the radius along its long axis, including the head of the radius
  2. Greatest width of the head of the radius
  3. Greatest width at the distal end of the radius
Carpals:
  1. Distance from the proximal articulation of the carpals with the radius/ulna to the distal articulation of the carpals with the metacarpals along the central axis of the limb
  2. Distance between the medial and lateral edges of the carpals at their greatest width along a line perpendicular to the central axis of the forelimb
Metacarpals:
  1. Length of each metacarpal along its long axis from its articulation with the carpals to the articulation with the proximal phalanx
  2. Width of each metacarpal at the midpoint of its long axis
Proximal phalanges (manus):
  1. Length of each proximal phalanx along its long axis
  2. Width of each proximal phalanx at the midpoint of its long axis
Intermediate phalanges (manus):
  1. Length of each intermediate phalanx along its long axis
  2. Width of each intermediate phalanx at the midpoint of its long axis
Distal phalanges (manus):
  1. Length of each distal phalanx along its long axis
  2. Width of each distal phalanx at the midpoint of its long axis
Femur:
  1. Length of the femur long axis, from the head to the condyles
  2. Greatest width of the head of the femur
  3. Distance from the medial margin of the medial condyle to the lateral margin of the lateral condyle
Tibia:
  1. Length of the tibia long axis
  2. Distance from the medial margin of the medial condyle to the lateral margin of the lateral condyle
  3. Greatest width at the distal end of the tibia at the articulation with the tarsal bones
Fibula:
  1. Greatest length of the fibula from the head to the distal tip of the lateral malleolus
  2. Greatest width of the head of the fibula
  3. Greatest width of the lateral malleolus
Tarsals:
  1. Distance from the proximal articulation of the tarsals with the tibia/fibula to the distal articulation of the tarsals with the metatarsals along the central axis of the limb
  2. Distance between the medial and lateral edges of the tarsals at their greatest width along a line perpendicular to the central axis of the hindlimb
Metatarsals:
  1. Length of each metatarsal along its long axis from its articulation with the tarsals to the articulation with the proximal phalanx
  2. Width of each metatarsal at the midpoint of its long axis
Proximal phalanges (pes):
  1. Length of each proximal phalanx along its long axis
  2. Width of each proximal phalanx at the midpoint of its long axis
Intermediate phalanges (pes):
  1. Length of each intermediate phalanx along its long axis
  2. Width of each intermediate phalanx at the midpoint of its long axis
Distal phalanges (pes):
  1. Length of each distal phalanx along its long axis
  2. Width of each distal phalanx at the midpoint of its long axis