# Evolution of the patella and patelloid in marsupial mammals

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## Abstract

22  The musculoskeletal system of marsupial mammals has numerous unusual features beyond the
23  pouch and epipubic bones. One example is the widespread absence or reduction (to a fibrous
24  "patelloid") of the patella ("kneecap") sesamoid bone, but prior studies with coarse sampling
25  indicated complex patterns of evolution of this absence or reduction. Here, we conducted an in-
26  depth investigation into the form of the patella of extant marsupial species and used the
27  assembled dataset to reconstruct the likely pattern of evolution of the marsupial patella. Critical
28  assessment of the available literature was followed by examination and imaging of museum
29  specimens, as well as CT scanning and histological examination of dissected wet specimens. Our
30  results, from sampling about 19% of extant marsupial species-level diversity, include new
31  images and descriptions of the fibrocartilaginous patelloid in *Thylacinus cynocephalus* (the
32  thylacine or "marsupial wolf") and other marsupials as well as the ossified patella in *Notoryctes*
33  'marsupial moles', *Caenolestes* shrew opossums, bandicoots and bilbies. We found novel
34  evidence of an ossified patella in one specimen of *Macropus rufogriseus* (Bennett's wallaby),
35  with hints of similar variation in other species. It remains uncertain whether such ossifications
36  are ontogenetic variation, unusual individual variation, pathological or otherwise, but future
37  studies must continue to be conscious of variation in metatherian patellar sesamoid morphology.
38  Our evolutionary reconstructions using our assembled data vary, too, depending on the
39  reconstruction algorithm used. A maximum likelihood algorithm favours ancestral
40  fibrocartilaginous "patelloid" for crown clade Marsupialia and independent origins of ossified
41  patellae in extinct sparassodonts, peramelids, notoryctids and caenolestids. A maximum
42  parsimony algorithm favours ancestral ossified patella for the clade [Marsupialia +

43  sparassodonts] and subsequent reductions into fibrocartilage in didelphids, dasyuromorphs and
44  diprotodonts; but this result changed to agree more with the maximum likelihood results if the
45  character state reconstructions were ordered. Thus, there is substantial homoplasy in marsupial
46  patellae regardless of the evolutionary algorithm adopted. We contend that the most plausible
47  inference, however, is that metatherians independently ossified their patellae at least three times
48  in their evolution. Furthermore, the variability of the patellar state we observed, even within
49  single species (e.g. *M. rufogriseus*), is fascinating and warrants further investigation, especially
50  as it hints at developmental plasticity that might have been harnessed in marsupial evolution to
51  drive the complex patterns inferred here.
52

## Introduction

54  Having diverged from the common ancestor of therian mammals during the late Jurassic period,
55  some 160 million years ago (Bi et al., 2014; Figure 1), marsupials are a diverse and biologically
56  fascinating group of mammals. A hallmark of marsupials is their developmental strategy:
57  marsupials have relatively short gestation periods, after which the newborn crawls to the teat for
58  a prolonged lactation phase, typically within a pouch (Alpin & Archer, 1987). As a result, the
59  embryo has delayed hindlimb development, and accelerated forelimb and cranial development
60  (Hamrick, 1999; Sánchez-Villagra, 2002; Garland et al., 2007; Sears, 2009). Despite the
61  constraints imposed by such as strategy (Garland et al., 2017), and geographical limitation to
62  Australasia and South America today, the over 350 extant species of marsupials exhibit great
63  diversity in their size, lifestyle, behaviour and anatomy (Walton and Richardson, 1989; Burgin et
64  al., 2018; Mammal Diversity Database, 2020).
65

66         One such diverse anatomical feature is the patellar sesamoid ("kneecap" or simply
67  patella). Sesamoids are skeletal elements found in connective tissues near joints (Vickaryous &
68  Olson, 2007; Abdala et al., 2019), that can modify the forces acting across it (e.g. acting as
69  levers; Alexander & Dimery, 1987; Fatima et al., 2019) and protect periarticular structures. In an
70  intriguing departure from the mammalian norm amongst monotremes and placentals, many
71  marsupials appear to lack a bony patella (Reese et al., 2001). Instead, many species possess a
72  region of fibrocartilage within the quadriceps femoris (QF) tendon at approximately the same
73  anatomical location as a patella, and which can be considered an unmineralised sesamoid
74  (Holladay et al., 1990; Reese et al., 2001; Inamassu et al., 2017; Abdala et al., 2019). This
75  fibrocartilage pad is commonly referred to as a 'patelloid'. There is great diversity in the
76  histological structure of the patelloid, including the size, degree of differentiation and structural
77  orientation of the fibrocartilage (Reese et al., 2001).
78

79         A previous high-level study of mammalian patellar evolution (Samuels et al., 2017)
80  indicated an unusual pattern of variation across marsupial families. Results suggested a single
81  evolutionary origin of an ossified patella within Metatheria (the larger stem-based group of
82  which marsupials are the only extant remnant), with instances of loss (to patelloid) in marsupials
83  and later reversion in some groups. However, Samuels et al. (2017) cautioned: "inferences about
84  the evolutionary history of the patella in Metatheria must remain tentative until further data
85  become available". Data on the form of the patella are absent for many marsupial species or rely
86  upon single observations or anecdotes within anatomical reports. There are also conflicting
87  reports as to whether certain species possess a patella, partially owing to the fibrocartilage
88  patelloid state not being recognised in all studies.

89
90      Knowledge of the form of the patella in marsupials remains patchy, with several gaps
91   waiting to be filled and incongruous statements requiring clarification. Throughout the literature,
92   certain species or more inclusive clades have been extensively studied, for example macropods
93   (kangaroos and kin), while others (e.g. many possums) have been poorly characterised. It is often
94   broadly generalised that all marsupials, besides bandicoots and the bilby, possess a fibrocartilage
95   patelloid instead of a bony patella (Reese et al., 2001; Vogelnest & Allan, 2015; Inamassu et al.,
96   2017). Earlier anatomical reports relied on direct observation to diagnose the presence of a bony
97   patella (Waterhouse, 1846; Osgood, 1921; Finch et al., 1989; Haxton, 1944; Johnson & Walton,
98   1989), but more recent studies have enabled better characteristation of patellar state through
99   radiography, histology and computed tomography (CT) (Holladay et al., 1990; Reese et al.,
100  2001; Inamassu et al., 2017; Vogelnest & Allan, 2015).
101
102     The aims of this study are twofold. Firstly, we seek finer clarification of the form of the
103  patella in marsupials at (and, where feasible, within) the species level. We attempt to fill gaps or
104  inconsistencies within literature data through new observations of extant species, plus the
105  recently extinct thylacine. Secondly, we combine literature reports and new observations, and
106  use this finer-level dataset to perform ancestral states reconstruction of the patella to address
107  lingering questions over its evolutionary pattern of gain, loss and possible regain. Specifically,
108  we test the hypothesis of Samuels et al. (2017) that a bony patella was ancestral for marsupials.
109
110

## Materials & Methods

112  Our extensive synthesis of the literature was followed by firsthand observations and photography
113  of osteological museum specimens, based on gaps in the data and availability of specimens for
114  study. CT scanning or x-ray radiography of a select number of specimens was then carried out,
115  dependent on the apparatus available, to test for the presence or absence of the patella.
116  Additionally, a small number of frozen stored macropod specimens were available for dissection
117  and histology of the QF tendon, described below. The combined literature and observational
118  findings were then used to reconstruct the evolutionary polarity of the patella in marsupials, with
119  character state codings following Samuels et al. (2017); described further below. Unfortunately,
120  in much of the literature and museum collections it was not clear whether specimens were wild-
121  caught, from zoos or otherwise, so we could not address this potential source of variation from
122  normal vs. abnormal biomechanical loads.
123

*Survey of Osteological Museum Specimens*

125  Marsupial osteological specimens were examined for grossly visible bone or dessicated
126  tendon/patelloid tissue. Specimens included those in collections held by the Natural History
127  Museum (NHMUK, London), the University Museum of Zoology, Cambridge (UMZC), the
128  Oxford University Museum of Natural History (OUMNH) and the National Museums of
129  Scotland (NMS, Edinburgh). Specimen details can be found in Table S4 of Supplementary Text
130  S1 (age and sex was generally unknown, so not included). Observations were only included for
131  specimens with adequate preservation of the QF tendon (so that true absence of patella/patelloid
132  could be discerned, versus possibility that these structures might have been cleaned off during
133  preparation). For some specimens, the nature of the patella was unclear by visual examination

134  alone. These specimens were selected for CT scanning or radiographic imaging, as described
135  below. For other specimens, if we saw obvious cartilage amidst the QF tendon (i.e. shiny
136  texture/different colour consistent with cartilage rather than collagenous tendon material) we
137  scored the specimen as having a patelloid. If a QF tendon was present, reasonably intact and
138  showed no sign of a swelling or other discontinuity consistent with a patelloid, we scored the
139  patella as absent. In cases where there was too little soft tissue remaining with the specimen (e.g.
140  most disarticulated femur-tibia-fibula bones), we refrained from scoring that specimen. This
141  relatively conservative approach considerably reduced our sample size but allowed for cautious
142  scoring, and we amended the sample size problem by as comprehensive a tour through major
143  United Kingdom zoological museum collections as we could manage within the time constraints
144  of the study.
145
146  *Computed Tomography (CT) Scanning and X-Ray Radiography of Osteological Specimens*
147  Micro-CT scans of 11 skeletal specimens were obtained and subsequently semi-automatically
148  segmented using Mimics (Materialise Inc., Leuven, Belgium) software. Scan details are in Table
149  S1 of Supplementary Text S1; patellar state coding results are in Table S5 of Supplementary
150  Text S1. Radiographs of seven skeletal specimens from NHMUK were also obtained. Details of
151  these radiographs are in Table S2 of Supplementary Text S1; patellar state coding results are in
152  Table S6 of Supplementary Text S1. The CT and radiographic images were used to diagnose the
153  presence or absence of an ossified patella within the QF tendon of the preserved specimens
154  imaged. In the case of CT scans, it was also possible to visualise soft tissue densities (using
155  thresholds set to cortical bone density) and therefore test for the presence of a (fibrocartilage)
156  patelloid. For ossified patellae, the thin cortical shell and internal trabeculae typical of an ossified
157  mammalian patella normally were visible, so scoring such specimens as "ossified" was simple.
158  For potentially fibrocartilaginous patelloids, CT scan or X-ray data helped illuminate the
159  composition of the QF tendon, beyond what was externally visible for the specimen. This was
160  aided by the typical presence of the lateral sesamoid (parafibula / "fabella" / "sesamoid bone of
161  Vesalli") proximal to the fibula (e.g. see figures in Results), as a gauge for the expected structure
162  and densities of an ossified sesamoid. If we observed a denser, structured unit within the QF
163  tendon in a position (proximal to the femoral condyles/tibia, in the middle of the QF tendon), we
164  scored it as a a fibrocartilaginous patelloid. If we saw no such striking heterogeneity within the
165  (relatively intact) QF tendon, we scored the patella as absent. Otherwise we omitted that
166  specimen from the study. Raw scan data are available at
167  https://figshare.com/s/c3f5c083910b31ae9363 (doi: 10.6084/m9.figshare.11627190).
168
169  *Computed Tomography of Frozen Intact Specimens*
170  In addition to the osteological specimens examined at museum sites, the study obtained four
171  recently deceased macropod specimens for medical CT scanning and histological examination.
172  These included one *Macropus rufus* and three *Macropus rufogriseus* specimens which had died
173  of natural causes at ZSL Whipsnade and London Zoos and had been stored frozen (-20°C). We
174  opportunistically examined these specimens in detail to test for variation within macropod
175  patellae. The hindlimbs of each specimen were scanned using CT prior to dissection. Details of
176  the scans are in Table S3 of Supplementary Text S1. Raw scan data are available at
177  https://figshare.com/s/c3f5c083910b31ae9363 (doi: 10.6084/m9.figshare.11627190).

178

*Dissection and Light microscopy of Frozen Specimens*

180 The right and left patellar tendons were harvested from two of the intact *Macropus rufogriseus*
181 (specimens 1 & 2). The tendons dissected from the thawed cadavers were fixed in 10% neutral
182 buffered formalin. Samples containing bone (diagnosed via imaging, above) were decalcified in
183 10% formic acid solution. All specimens were sectioned in the sagittal plane along the midline
184 and directly lateral to the midline. The tendon sections were dehydrated and embedded in
185 paraffin wax blocks. Microtome sections were cut between 4 and 6 μm. Sections were stained
186 with Haematoxylin and Eosin, and Masson's trichrome. In the case of tendons where ossified
187 patellar tissue was found to be present, sections were also stained with Safranin O/Fast Green for
188 the identification of cartilage, and Von Kossa to highlight the presence of calcium salts. The
189 histological sections were examined by light microscopy and images obtained via scanning at
190 high resolution using a Leica SCN400F scanner.

191

192 *Evolutionary Reconstructions of Ancestral State*

193 Standard phylogenetic character mapping methods were used to reconstruct the evolutionary
194 polarity of the patella in marsupials. A patellar character score was assigned to each species for
195 which data had been obtained on the basis of the findings gathered from the literature, direct
196 observations, radiographic/CT imaging and light microscopy; totalling 94 species. The patella
197 was coded as absent (score = 0), fibrocartilage patelloid (score = 1) or ossified (score = 2) for
198 each species; and one additional "crown Eutheria" outgroup was scored as state 2; all following
199 Samuels et al. (2017). In species where more than one state had been observed, or conflicting
200 reports in the literature were unresolved, both possible states were included. For example,
201 *Macropus rufogriseus* was coded as 1/2 because both patelloid and ossified patella states were
202 observed (see Results). A composite phylogenetic tree (here referred to as our "original tree")
203 containing the species for which patellar data were available was obtained from the Timetree
204 database, based on current literature (http://timetree.org; Hedges et al., 2006; see also May-
205 Collado et al., 2015), with fossil outgroups added from Forasieppi (2009) and Bi et al. (2014,
206 2018). The data matrix is in Supplementary Data S1. We also conducted a sensitivity analysis of
207 the assumed phylogeny by modifiying the fossil outgroups to match those adopted in Samuels et
208 al. (2017).

209

210      The evolutionary history of the patella was reconstructed over the phylogenetic tree using
211 the maximum likelihood (Mk1) model in Mesquite software (Maddison & Maddison, 2017),
212 with branch length calibrations following Samuels et al. (2017); unavailable branch
213 length/divergence time data for extant taxa used the assumption that branch lengths were one-
214 half those connected to the next most deeply nested node. For comparison, a second
215 reconstruction was generated using the maximum parsimony algorithm in Mesquite, with
216 unordered character states; although we also checked this analysis by changing the
217 reconstruction method to ordered character states (i.e. preferring stepwise changes from state 0 to
218 1 to 2 or the converse; not 0 to/from 2). We also re-ran analyses switching state "0" (no patella)
219 coded fossil taxa to state "0/1" (patelloid possible) to check sensitivity to the vagaries of
220 preservation and interpretation of missing fibrocartilages.

221

222

## 223 Results

224  A comprehensive literature review was the starting point for this study (Supplementary Text S1:
225  Table S7). To summarise: those higher-level clades for which there was evidence supporting the
226  presence of an ossified patella (state 2) included Caenolestidae, Notoryctidae, Thylacomyidae
227  and Peramelidae, with the possibility of an ossified patella in Tarsipedidae (Waterhouse, 1846;
228  Osgood, 1921) and the quokka *Setonix brachyurus* (Dawson et al., 1989; scored as state 1 or 2
229  due to ambiguity in specimens available). Even in the well-studied taxon *Didelphis virginiana*
230  we found much confusion in the literature regarding patellar state, forcing us to code it
231  ambiguously as state 1 or 2. In a small number of species, including Vombatidae and
232  Phascolarctidae, the patella was noted as absent (state 0) in some studies and in the form of a
233  patelloid (state 1) in others. For example, in wombats, published descriptions ranged from
234  absence of any sesamoid (Waterhouse, 1846; Lee & Carrick, 1989) to an equivocal 0/1 character
235  state (Home, 1808; Vogelnest and Allan, 2015) to presence of a patelloid (Macalister, 1870). The
236  remainder of species for which data existed were scored as having a patelloid.
237
238        The most critical areas where data were sparse include the many South American
239  opossums (excluding the genus *Didelphis*; only ~7% of diversity), Microbiotheriidae (uncertain
240  coding; see below), some species of Dasyuromorphia (e.g. only ~10% of Dasyuridae), and the
241  small Australian opossums and gliders (Acrobatidae, Tarsipedidae, Petauridae, Phalangeridae
242  and Pseudocheiridae; uncertain coding for the former two and ~6-25% diversity scored for the
243  latter three). Overall, our final data matrix (see https://figshare.com/s/c3f5c083910b31ae9363
244  (doi: 10.6084/m9.figshare.11627190)) coded for about 72 species of marsupials, ~19% of total
245  diversity (Figure 2; Burgin et al., 2018; Mammal Diversity Database, 2020). Nonetheless, we
246  sampled at least one species each from all family-ranked clades of extant Marsupialia; even
247  about one-third of the relatively species-rich Macropodidae.
248
249  *Observations and Imaging of Osteological Specimens*
250  Firsthand observational data from preserved osteological specimens are in Table S4 of
251  Supplementary Text S1. The nature of the patella was recorded for each specimen observed at
252  the four museum collections visited. Details of specimens where CT or radiography was used for
253  confirmation of patella mineralisation status are in Tables S5 and S6 of Supplementary Text S1.
254  Examples of photographs and scans obtained are in Figures 3-6, with examples of radiographs in
255  Figure 7.
256
257        Most findings from specimen-based examinations supported those from the literature
258  review. Although it was not possible to conclusively confirm the presence of fibrocartilage
259  without histological samples, the visual and imaging observations supported the presence of a
260  fibrocartilage patelloid within the QF tendon for the majority of both Australian and American
261  marsupials (Figures 3, 4). CT scans confirmed our visual observations for numerous marsupials
262  (e.g. Figure 4), demonstrating relatively low density and homeogeneous structure of the QF
263  tendon and patelloid (Figures 3, 5) vs. the ossified patella or other bones (Figure 6). Our analysis
264  supported the literature in finding likely patelloids in the dessicated QF tendons of both
265  hindlimbs of a well-preserved specimen of the now-extinct *Thylacinus cynocephalus* (Figure 5).
266  The form of the patella of this species was briefly mentioned as a patelloid by Cunningham
267  (1882) and Wood (1924), which Warburton et al. (2019) supported based on a more exhaustive
268  study (also character coding in Forasieppi (2009)). We thus accepted these character state

269   codings where available evidence best supported interpretation as fibrocartilage patelloid vs.
270   alternatives (also see Methods). Importantly, observations were made in numerous species for
271   which there had previously been no data recorded. Unfortunately, there was still a large number
272   of species for which we observed no specimens or found no clear literature descriptions; our
273   sampling of Marsupialia was necessarily incomplete (see sampling summary above).
274
275        Ossified patellae were evident in Caenolestidae, Notoryctidae, Thylacomyidae and
276   Peramelidae (Figure 6), which also supported the literature review. The structure of the patella in
277   these taxa is similar and also similar to that in other mammals (Samuels et al., 2017), consistent
278   with common developmental mechanisms (e.g. endochondral ossification). We have provided
279   among the first detailed images (external and internal morphology) of the ossified patella in these
280   taxa, in addition to the patelloids in others.
281
282   *Computed Tomography and Visual Observations of Frozen Specimens*
283   CT scans of the wet *Macropus rufogriseus* and *Macropus rufus* specimens revealed the presence
284   of an ossified patella in the left hindlimb of one *Macropus rufogriseus* (specimen 1; Figure 8).
285   The basic structure of this patella that was evident in CT images (e.g. cortical shell, trabecular
286   interior) matched the structure evident from CT scans of other marsupials with ossified patellae,
287   or the structure of the lateral sesamoid (Figures 3, 6, 8). Upon subsequent dissection, this ossified
288   patella was clearly evident (Figure 8). In the remaining three specimens of *Macropus*, a patelloid
289   was observed on the CT scans and subsequently confirmed to be fibrocartilage in one specimen
290   (specimen 2) by dissection and histological examination (Figure 9).
291
292   *Light Microscopy of Frozen Specimens*
293   Histological examination confirmed the presence of an ossified patella in the left QF tendon of
294   specimen 1 of *Macropus rufogriseus* (Figures 10, 11). The majority of the patella was composed
295   of cancellous bone, with cortical bone comprising the superficial third (Figure 11A);
296   approximately typical for an ossified mammalian patella. The Safranin O/Fast Green staining
297   highlighted a layer of articular hyaline cartilage covering the deep surface: the surface in
298   articulation with the femur. Staining with Von Kossa identified the presence of calcium salts,
299   with a central region of increased calcium density. In contrast, the right QF tendon of specimen
300   1, and both QF tendons of specimen 2, contained a typical fibrocartilage patelloid (Figure 10).
301   The presence of cartilage was best highlighted by staining with Safranin O/Fast Green, while
302   collagen fibres were most clearly illustrated with Masson's trichrome. As expected, the majority
303   of the patelloid stained poorly with Von Kossa, illustrating that the tissue was not ossified
304   (Figure 11B). However, stain accumulated in one small region in the centre of the tendon,
305   suggesting a small amount of nascent mineralisation/ossification there.
306
307   *Evolutionary Reconstructions*
308   Figure 12 illustrates the reconstructed evolution of the patella in marsupials, according to the
309   maximum likelihood (Mk1) and parsimony algorithms. Under the maximum likelihood model,
310   absence of any patellar sesamoid (mineralised or patelloid) was reconstructed as the most likely
311   ancestral state for Metatheria. Sparassodonts, as in Samuels et al. (2017), were united by an
312   apparently independently evolved ossification of the patella (e.g. Sinclair, 1905, Wood, 1924;
313   Argot, 2004; Forasieppi, 2009). Our reconstruction indicates that the common ancestor at the

314    root of the crown group marsupial tree was most likely (62%; greater at deeper nodes 3-6 in
315    Figure 12) to have evolved a fibrocartilage patelloid (i.e. character state 1), which has been
316    maintained in the majority of sampled marsupial species. The likelihood of an ancestral patelloid
317    state in marsupials increased (to 97%; same if using the Samuels et al. (2017) tree) if a patelloid
318    state was considered possible in all non-marsupial metatherians lacking ossified patellae (e.g.
319    *Herpetotherium* coded 0/1 rather than 0). We found three separate instances of evolution of an
320    ossified patella (i.e. character state 2) from the ancestral patelloid within the crown group,
321    occurring at the nodes for Caenolestidae, Notoryctidae and Peramelemorphia, according to the
322    maximum likelihood reconstruction (with our original tree and that of Samuels et al. (2017)).
323    Hence an ossified patella may have evolved at least four times in major clades within Metatheria.
324
325        There was also one apparent instance of loss of a patelloid (i.e. reversal to character state
326    0), in *Dromiciops* (Microbiotheriidae), however this was only suggested by observations from a
327    single study (Szalay & Sargis, 2001). Similarly, for the closely-related Vombatidae and
328    Phascolarctidae, and for *Sarcophilus harrisii* and *Metachirus nudicaudatus*, some sources noted
329    complete absence of any patella or patelloid within the quadriceps tendon (full details in
330    Supplementary Text S1). This evidence leads to the tentative speculation that a reversal from
331    patelloid to absence of any sesamoid occurred more than three times in our sample of
332    Marsupialia, although our coding for this character state was left equivocal (state 0 or 1; Figure
333    12), as some more recent sources imply that a patelloid may indeed be present. Indeed,
334    dissections of two male and two female adult koalas (*Phascolarctos cinereus*) indicated the
335    bilateral presence of patelloids, not ossified patellae (Hazel Richards, Monash University, pers.
336    comm. 2019; confirmed by CT scans), as shown in Figure 13. There were several instances of
337    ossified patellae being observed in (individuals of) a single species, despite other species within
338    the same family-rank clade possessing a fibrocartilage patelloid. These included *Didelphis*
339    *virginiana*, *Tarsipes rostratus* and multiple macropod species (e.g. *Setonix brachyurus*).
340    Likewise, here, we coded these states as equivocal until stronger sampling can be conducted.
341    Regardless, transformations from an unossified patelloid to patella appeared common in our
342    maximum likelihood analyses for Marsupialia.
343
344    In comparison with our maximum likelihood analyses, maximum parsimony analysis with
345    unordered character states (Figure 12; branch colours) presented a very different evolutionary
346    pattern. An ossified patella united most of Metatheria [Sparassodonta + Marsupialia] as an
347    ancestral state. Next, there were three independent reductions to a patelloid (i.e. no ancestral
348    transformations from patelloid to patella for major clades) along the lineages to Didelphidae,
349    Dasyuridae/Dasyuromorphia and Diprotodontia. Otherwise the results were similar. Forcing the
350    character states to evolve in an ordered regime produced results more concordant with the
351    maximum likelihood analysis: ancestral fibrocartilage for [Sparassodonta + Marsupialia], then
352    two independent origins of ossified patellae in sparassodonts and caenolestids, although it was
353    ambiguous whether ossified patellae were homologous or homoplastic for [Dasyuridae +
354    Peramelemorphia + Notoryctidae] on the tree used, involving one or two more origins of an
355    ossified patella, totaling 3-4 occurrences. Using a phylogeny concordant with that of Samuels et
356    al. (2017) produced identical results for both parsimony models (unordered/ordered).
357
358    When we coded all fossil taxa originally scored as having "patella absent" (= state 0) to "patella
359    absent"/"patelloid present" (= state 0/1) under maximum parsimony, we obtained some similar

360  results to those before this change of assumptions. Unordered parsimony produced an ambiguous
361  state 1/2 (patelloid/ossified patella present); not 2; at the node [Sparassodonta + Marsupialia],
362  then equivocal states for some of the more inclusive branches within Marsupialia, so 2-4 total
363  independent ossification events could have occurred. Ordered parsimony produced the same 3-4
364  origins of an ossified patella as above. Conducting this same procedure on the Samuels et al.
365  (2017) tree, again we found ambiguity under unordered parsimony, with state 1/2 prevailing up
366  to [Sparassodonta + Marsupialia], then an uncertain state 1/2 across major nodes until
367  Diprotodontia (state 1); leaving it uncertain whether an ossified patella evolved independently or
368  not in caenolestids, Notoryctidae, or Peramelemorphia (Figure S1). Yet again, ordered
369  parsimony gave results concordant with others using that assumption.
370
371  Overall, then, depending on which tree and evolutionary reconstruction method was adopted, we
372  tended to obtain 3-4 origins of an ossified patella in Sparassodonta + Marsupialia. Nonetheless, it
373  remained feasible that an ossified patella might have reversed to fibrocartilage 3 times (with
374  unordered parsimony and our original tree and character scores).
375

## Discussion

377  We conducted an in-depth analysis of the form of the marsupial patella across Marsupialia, using
378  observations from both osteological and wet specimens, combined with critical analysis of the
379  available literature (Table S7 of Supplementary Text S1). Our finer-level dataset did not clearly
380  support the hypothesis of an ancestrally-ossified patella in marsupials, proposed by a previous,
381  less detailed study also using maximal likelihood character mapping methods (Samuels et al.,
382  2017). The new maximum likelihood ancestral state reconstruction indicated evolution of an
383  ossified patella from a fibrocartilage patelloid at three separate instances during marsupial
384  evolution. However, our parsimony-based analysis contradicted this reconstruction in that an
385  ossified patella evolved first in Metatheria, and then was independently transformed into a
386  patelloid at least three times; altered if we assumed parsimony with ordered character polarity.
387  Our conclusions thus hinge on which evolutionary algorithm is preferred. Regardless, our
388  findings concur that there is extensive homoplasy for the ossified patellar sesamoid in
389  Metatheria.
390

391  We have also discovered the presence of an ossified patella in one individual of
392  *Macropus rufogriseus*, a species previously stated to possess a fibrocartilage patelloid(Reese et
393  al., 2001). Intriguingly, this *M. rufogriseus* individual exhibited two different patellar states: the
394  right knee had a fibrocartilage patelloid, as anticipated from the literature, whilst the left had a
395  very well-differentiated bony patella complete with articular hyaline cartilage. The discrepancy
396  within these species, and particularly within an individual, may go some way towards explaining
397  apparent contradictions in literature data. This may, in part, be due to the vague references found
398  in certain older studies. However, it also raises the possibility that several marsupial species,
399  previously thought to possess a patelloid, are actually able to develop an ossified patella, perhaps
400  in different environments (e.g. mechanical, developmental) or simply due to random variation.
401  The fact that different patellar states exist among closely related species, or even within
402  individuals, highlights the complexity of marsupial patellar evolution. Perhaps developmental
403  potential for an ossified patella may exist in most or all marsupial species, with ossification only
404  actually occurring in certain cases.
405

406     The results presented above challenge the frequently quoted statement that "all
407 marsupials, except bandicoots and the bilby" lack an ossified patella (Reese et al., 2001;
408 Inamassu et al., 2017; Vogelnest & Allan, 2015). The very clear presence of a bony patella in the
409 Caenolestidae and Notoryctidae (e.g. Thompson & Hillier, 1905; Figure 6) is sufficient evidence
410 to the contrary. Furthermore, the observation of an ossified patella in a single *Macropus*
411 individual, combined with some ambiguity in literature noted above and in the Supplementary
412 Text S1 (Table S7), cautions against the use of broad cross-species generalisations which
413 implicitly assume 100% evolutionary fixation of character states within/between species. Sarin et
414 al. (1999) examined the incidence of two sesamoids (the fabella and os peroneum) in primates,
415 finding intra-species variation in the occurrence of the former sesamoid (vs. quasi-constancy of
416 the latter), and inferring a decline in its incidence along the primate stem lineage and then
417 secondary increase in *Homo*. Berthaume et al. (2019) then found remarkable increases in the
418 ossification of the fabella over the past 150 years in human populations, attributing this to
419 improved health and nutrition favouring larger body sizes and thus increased mechanical stimuli.
420
421     Our results indicate a complex pattern of evolution and development of this sesamoid,
422 with patellar states apparently not constant in all clades, and with certain individuals capable of
423 developing an ossified patella in species otherwise typically possessing a patelloid. Thus, there is
424 polymorphism in several marsupial taxa, as hinted at by Samuels et al. (2017), although the
425 biological mechanisms underlying this variation remain uncertain—and certainly deserving
426 deeper mechanistic studies. A genetic basis is hypothesised for many sesamoids, with further
427 development driven by epigenetic factors (see Abdala et al., 2019 for recent review). Repeated
428 evolutionary losses and regains of an ossified patella are thus very plausible for marsupials,
429 perhaps through evolutionary 'maintenance' of a transitional structure like the patelloid.
430 Marsupials are known to have apomorphically delayed hindlimb development (Hamrick, 1999;
431 Sánchez-Villagra, 2002; Garland et al., 2007; Sears, 2009) compared with other mammals, so we
432 speculate that this heterochronic shift might have also impacted patellar development and its
433 evolution. New data on patellar development in marsupials are urgently needed, especially in
434 light of novel recent insights into the tissue origins and molecular controls of patellar
435 development in mammals (Eyal et al., 2015,2019; Márquez-Flórez et al., 2018; Samuels &
436 Campeau, 2019).
437
438     The maximum likelihood reconstruction of Samuels et al. (2017) suggested a single
439 origin of a bony patella in Metatheria, prior to the divergence of Marsupialia and Sparassodonta,
440 followed by reduction to a fibrocartilage patelloid in most marsupials (e.g. ancestral
441 Diprotodontia) and re-ossification in some deeper lineages (e.g. Tarsipedidae). This was found to
442 be more likely than multiple instances of bony patellar evolution (Samuels et al., 2017). Our
443 present study focused on data at the species level and much more expansive sampling of
444 individual specimens (Supplementary Text S1; Tables S1-S7), rather than just family-level
445 clades as in Samuels et al. (2017); and we placed Caenolestidae and Didelphidae as sister taxa in
446 our phlogeny (Figs. 2, 9) rather than successive branches (as per our source of the phylogeny; see
447 Methods). Alternative phylogenies (e.g. Bininda-Emonds et al. 2007; Meredith et al., 2011;
448 O'Leary et al., 2013) would alter our results at least slightly, and resolution of this issue requires
449 better consensus among mammalian phylogenies in general. We found conflicting evolutionary
450 patterns that only concur with Samuels et al.'s (2017) if maximum parsimony (with unordered
451 character states) rather than likelihood (or ordered character states) is adopted. In the latter case,

452  maximum likelihood (and ordered-state parsimony) favoured the more conventional scenario that
453  Metatheria lacked an ossified patellar sesamoid ancestrally, but may have possessed a patelloid
454  (the origin of which is dependent on coding in extinct non-marsupial metatherians). Later, three
455  or more different marsupial clades independently ossified the patelloid that was ancestral for the
456  crown group. Resolving this discordance will depend upon what algorithm is favoured (we
457  suggest that the latter results are the most plausible), but also on acquiring more high-resolution
458  data across Metatheria.
459
460        We, however, contend that coding the patellar states as ordered is more plausible given
461  available ontogenetic data and mechanobiological theory (e.g. Sarin & Carter, 2000; Márquez-
462  Flórez et al., 2018; Abdala et al., 2019). Reciprocally, the general congruence between those
463  results and maximum likelihood offer some reassurance that an evolutionary sequence from
464  little/no patella to "patelloid" to 3+ parallel evolutions of bony patellae may be the most
465  reasonable conclusion at present. Furthermore, maximum likelihood analyses tend to be favoured
466  for studies of character evolution (Schluter et al., 1997; Pagel, 1999; Oakley, 2003). Nonetheless,
467  this complex question of patellar evolution in metatherians, which has long been plagued by a
468  seeming bias toward simple answers, deserves continued and deeper inquiry. There are
469  interesting broader stakes for evolutionary developmental biology and biomechanics. For
470  example, marsupial patellae are relevant to the concept of "traction epiphyses", that 'new' bones
471  may originate by disconnection from or be 'lost' by fusion to long bones (see reviews in
472  Vickaryous and Olsen, 2007; Abdala et al., 2019). Whether ossified or fibrocartilaginous, the
473  patella of marsupials and other mammals might have originated as a traction epiphysis from the
474  femur (Eyal et al., 2015, 2017) or the tibia. In cases where the patella was entirely lost, it might
475  have even merged with one of those bones. Sufficient developmental data do not, to our
476  knowledge, exist to enable testing of this idea at present.
477
478        Unfortunately, several species were unavailable for inclusion in this study, and
479  occasionally only single specimens of a particular species were observed. Further data on the
480  form of the patella in marsupials and their metatherian cousins are still required, in order to
481  further clarify the pattern of patellar evolution in marsupials. Additionally, the age (or even gross
482  ontogenetic stage; other than near-adult) of the preserved specimens studied was largely
483  unknown. Previously, Szalay & Sargis (2001) had suggested that ossification may occur in older
484  individuals of some species, referring specifically to *Didelphis virginiana*. However,
485  examination of an ontogenetic series of specimens, perhaps of multiple species, would be
486  required to resolve this matter. Such an age-related correlation is supported by the prevalence of
487  fabellar sesamoid ossification in humans (Berthaume and Bull, 2020), for example.
488
489        The (tentative) conclusion that a bony patella evolved, or was reacquired, at multiple
490  times in divergent marsupial species raises interesting questions regarding the function of this
491  sesamoid. Previous studies examining the form of the marsupial patella have varied in their
492  conclusions about how the presence of a patella or patelloid relates to the ecology and behaviour
493  of the species studied. Holladay et al. (1990) attributed the presence of a patelloid in macropods
494  to their pattern of locomotion. In contrast, Reese et al. (2001: p.293) challenged that "the lack of
495  a bony patella is typical for marsupials", but the different patelloid "types" they observed result
496  from the different mechanical stresses acting on the knee joint of different species. Abdala et al.
497  (2017) suggested that the presence of a fibrocartilaginous patella in some frog species might be

498  correlated with jumping biomechanics, mirroring some speculations from studies of the
499  fibrocartilaginous patelloid in marsupials (Holladay et al., 1990; Reese et al., 2001). However, to
500  the degree that a fibrocartilaginous patelloid is present in marsupials, it is a plesiomorphic trait
501  for lineages that hop, rather than co-evolving with hopping gaits; hence we doubt this correlation
502  for marsupials. We did not carry out functional analysis of the marsupial patella here; however,
503  the observations made here certainly warrant future functional studies. In particular, the question
504  of why would an ossified patella form in some individuals of a species, while others possess a
505  fibrocartilage patelloid, remains unresolved and would require quantitative biomechanical
506  analyses to test. This is an exciting foundation for future potential studies in evolutionary
507  developmental biomechanics.
508
509

## Conclusions

511      New, finer-scale evolutionary reconstructions presented here suggest that an unossified
512  patelloid was ancestral for marsupials, with repeated gains (three or more) of an ossified patella
513  within crown group Marsupialia. Our novel observation of an ossified patellae in an individual of
514  *Macropus rufogriseus* challenges the typical generalisation regarding the fibrocartilage nature of
515  the marsupial patelloid. However, a large number of species remain unstudied, including several
516  small species of possum, for which preservation of the patella is difficult to ascertain or ensure in
517  museum specimens. Furthermore, the mechanistic basis of of patellar sesamoid "evo-devo" and
518  biomechanics remains an important open question for explaining how and why patellar forms
519  changed repeatedly across metatherian evolution.
520
521

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531
532

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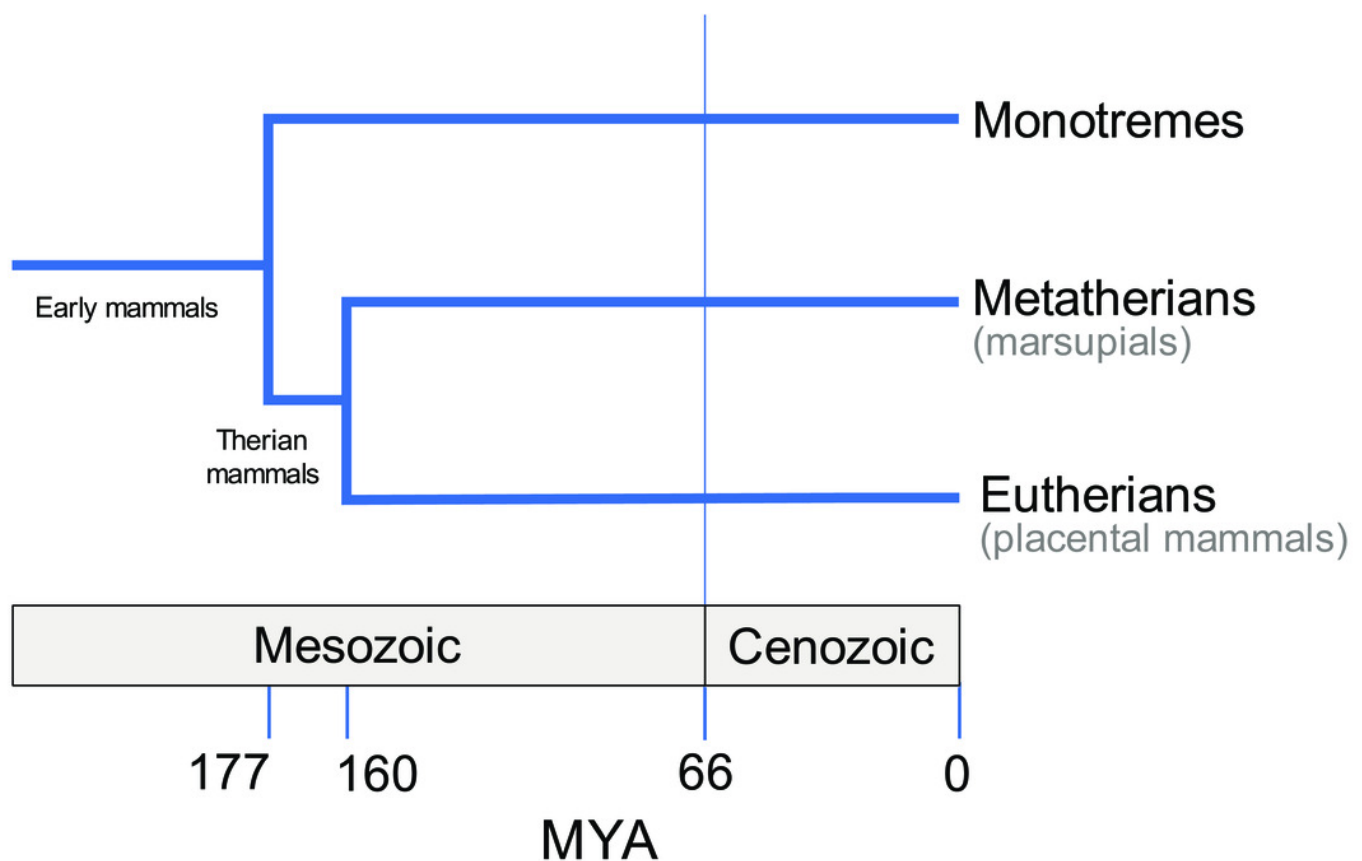
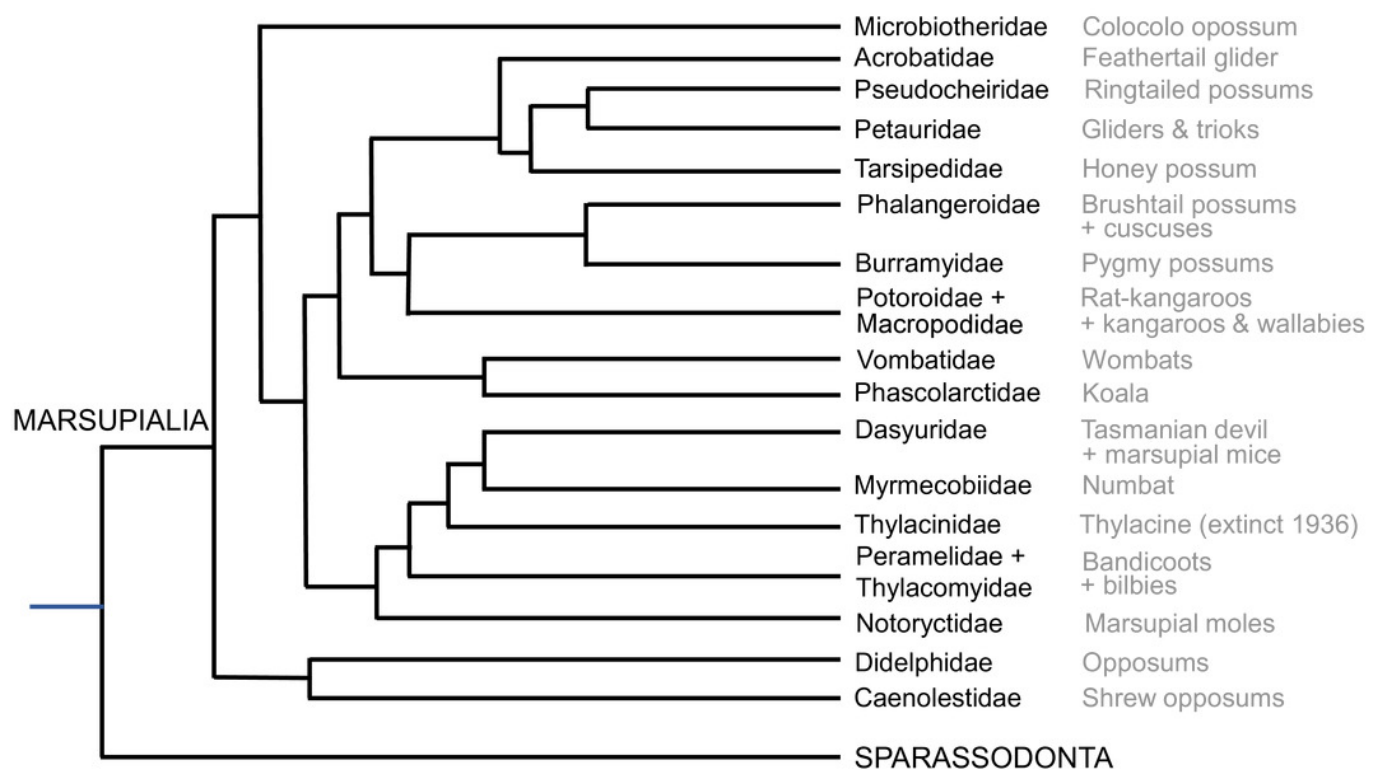
# Figure 1

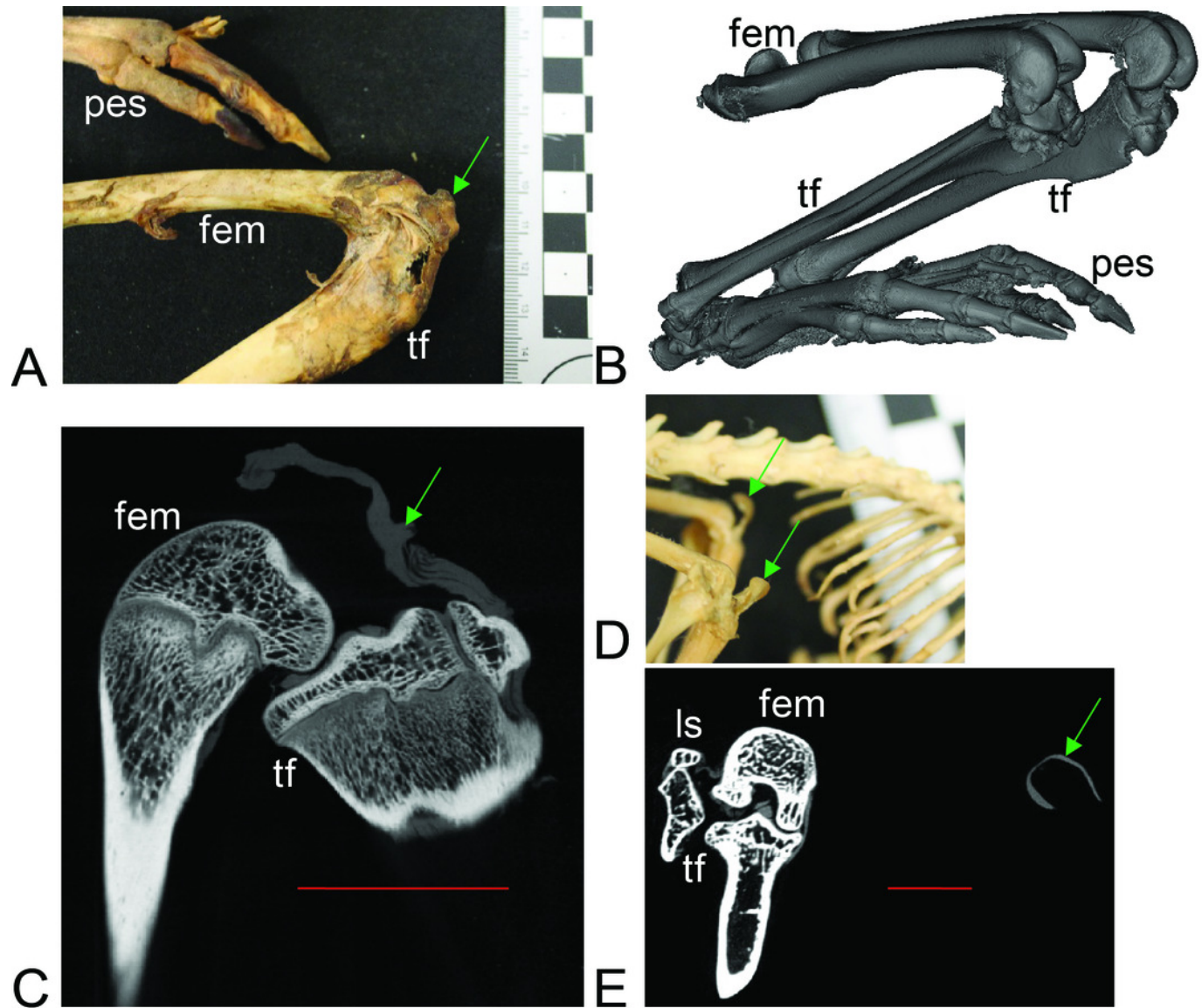
Evolutionary divergence within Mammalia (e.g. May-Collado et al., 2015).

Approximate timings in MYA (million years ago). Divergence time estimates from Timetree database (http://www.timetree.org; Hedges et al., 2006).

# Figure 2

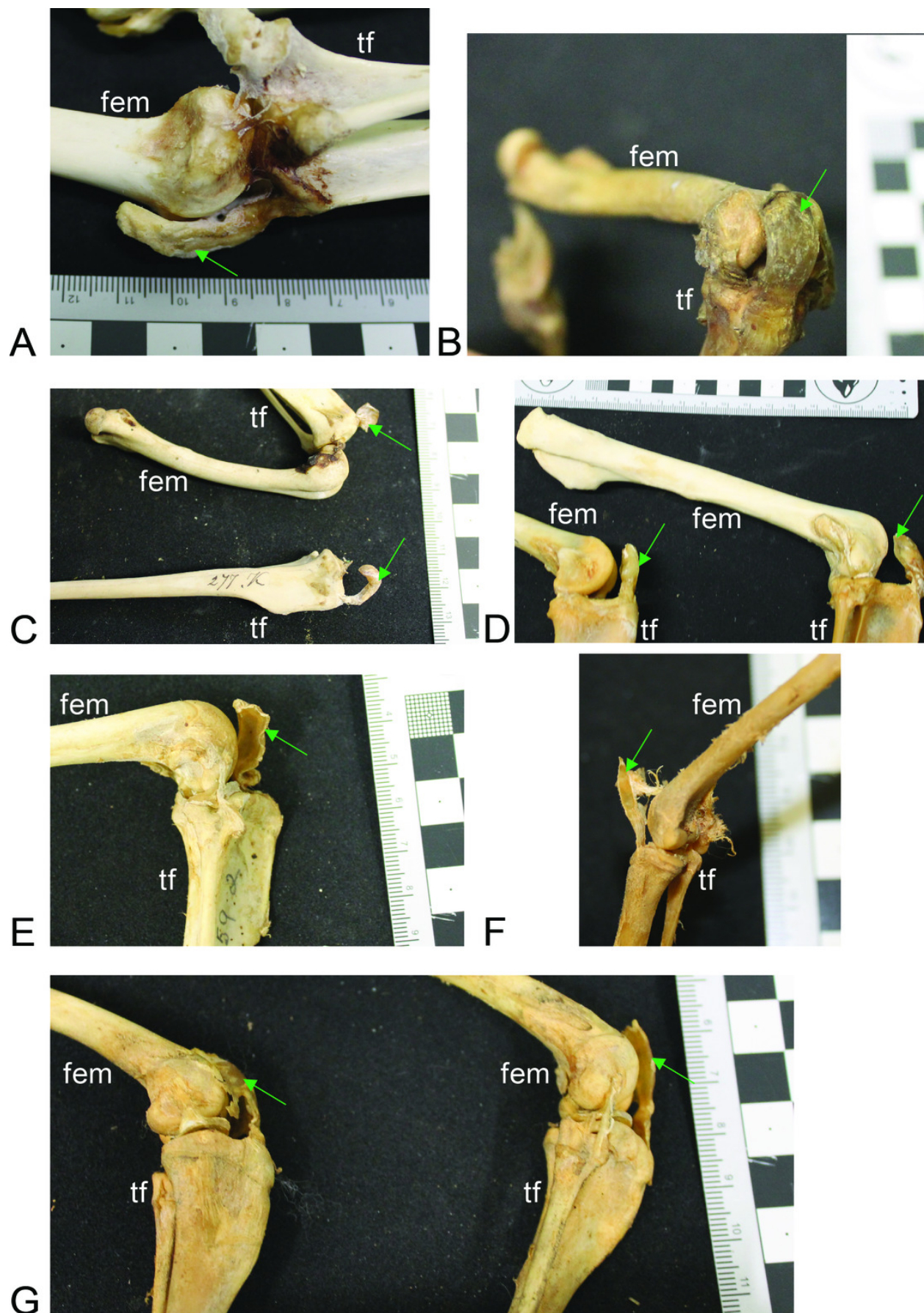
Summary cladogram of the main extant marsupial family-rank clades sampled in this study.

Evolutionary relationships are according to the Timetree database (http://www.timetree.org; Hedges et al., 2006).

# Figure 3

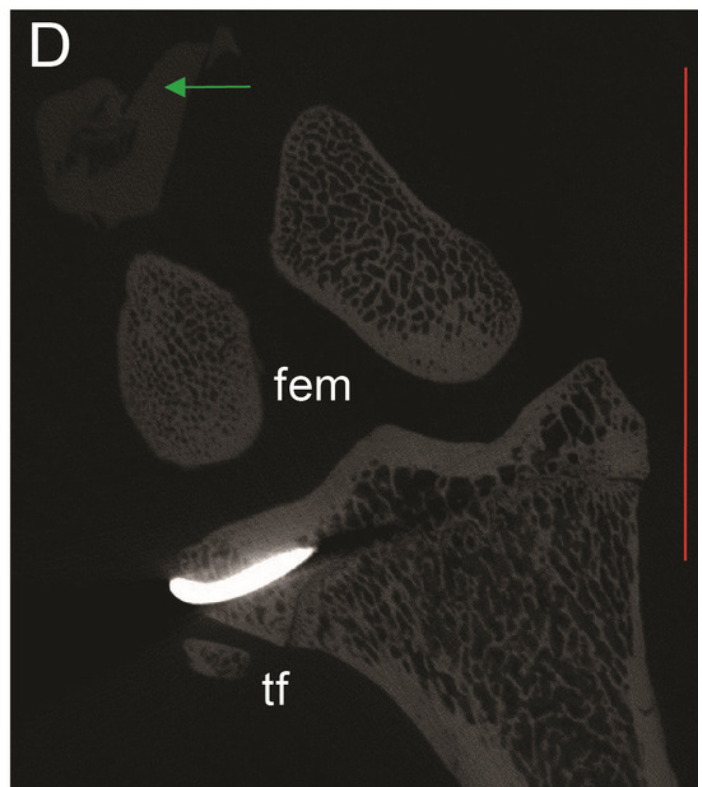
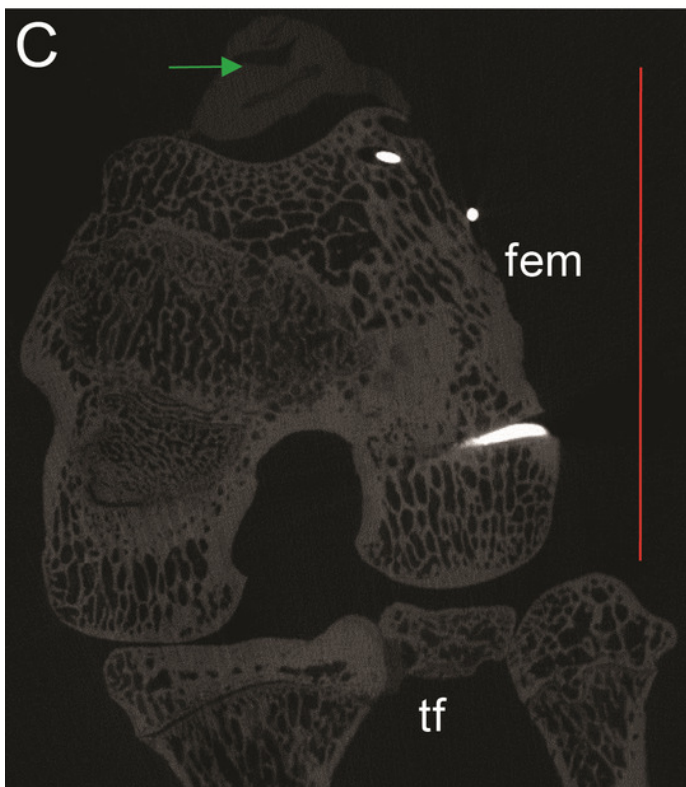
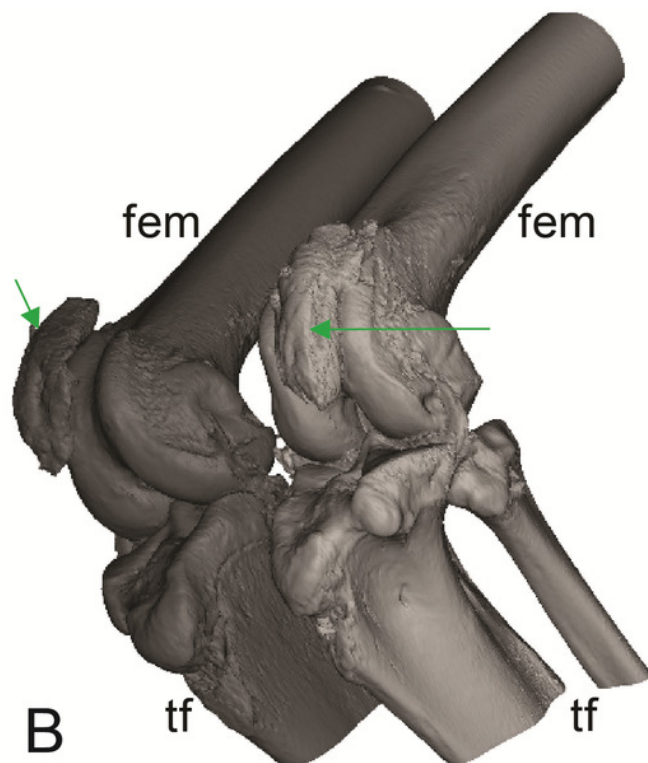
Example images of marsupial specimens with patelloids (Supplementary Text S1: Tables S1,S5).

(A, B, C): *Thylogale billiardierii* (UMZC specimen A12.50-3) pademelon -- no ossified patella as shown in photograph in A (scale bar squares = 1 cm), 3D reconstruction from micro-CT scans of hindlimbs in B and longitudinal section from micro-CT scans with grey (fibro)cartilage density corresponding to patelloid in C. (D,E): *Petaurus breviceps* (UMZC specimen A9.40-2) sugar glider. (D) Skeleton in right side view showing left and right patelloids. (E) Micro-CT scan of hindlimbs in frontal plane view showing (on left) right hindlimb/knee joint and (on right) QF tendon/patelloid. Green arrows point to location of patelloid where visible. Note lower density of QF tendon and patelloid (greyer shades) vs. bony tissue (whiter shades) in C and E. Labels: "fem" = femur; "ls" = lateral sesamoid; "pes" = pes (hindfoot); "tf" = tibia and fibula. Red horizontal scale bars in C and E are 20mm and 4mm.

# Figure 4

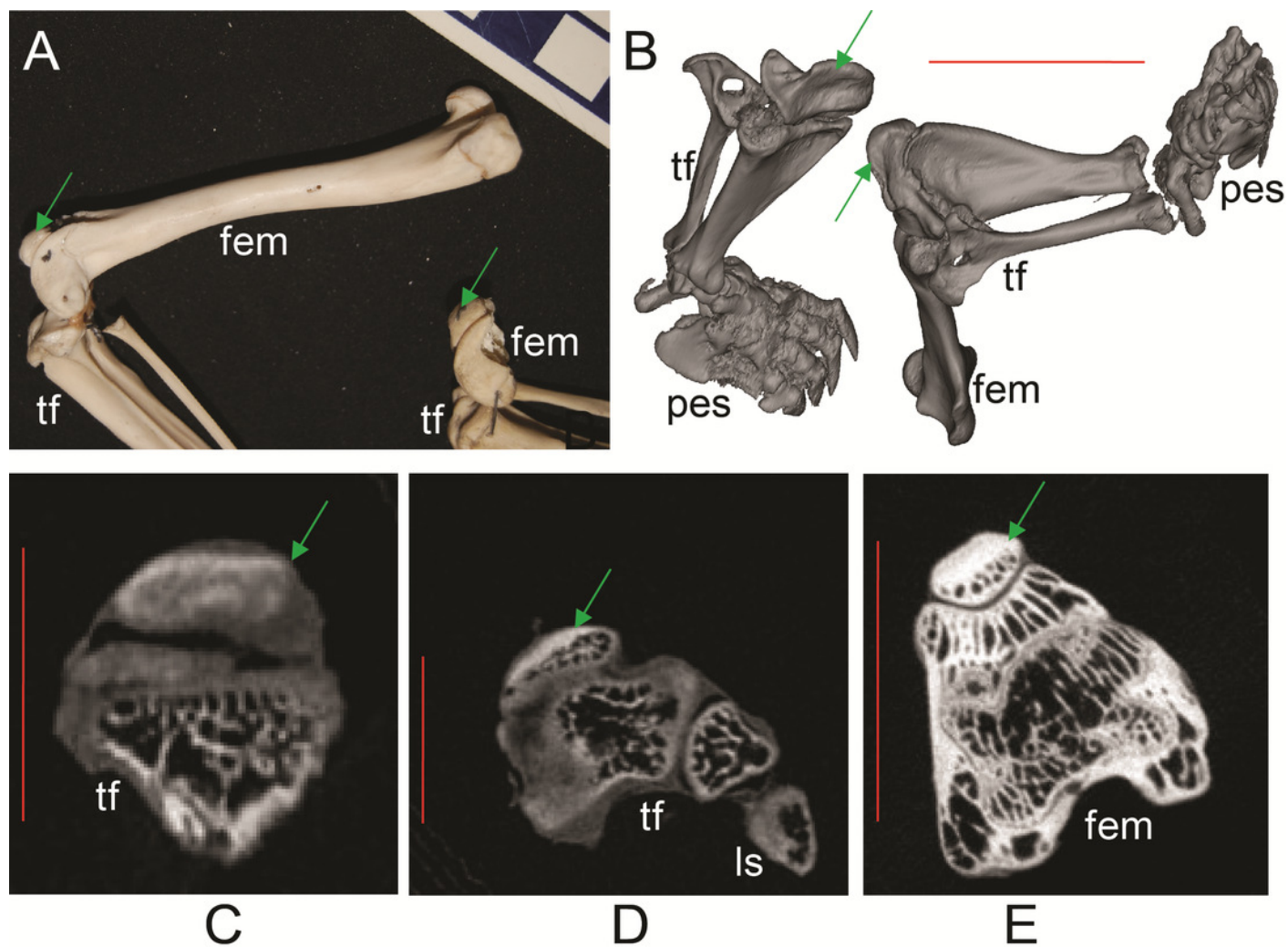
Other example images of marsupial specimens with patelloids (Supplementary Text S1: Table S4).

(A) *Vombatus ursinus* (NHMUK specimen 196.4.6.29.1) wombat, left hindlimb. (B) *Trichosurus vulpecula* (NHMUK specimen 89.266) brushtail possum, left hindlimb. (C) *Bettongia lesueur* (NHMUK specimen 277.70) boodie, right (top) and left (bottom) hindlimbs. (D) *Macropus agilis* (NHMUK specimen 70.368) agile wallaby, left and right hindlimbs. (E) *Onychogale fraenata* (UMZC specimen A12.59/2) bridled wallaby, right hindlimb. (F) *Dasyurus viverrinus* (UMZC specimen A6.11-6) quoll, right hindlimb. (G) *Aepyprymnus rufescens* (UMZC specimen A12.77-3) rufous bettong, left and right hindlimbs. Green arrows point to location of patelloid where visible. Scale bar squares = 1 cm; note some photos are at oblique angles to scale and hence not precisely to-scale. Labels: "fem" = femur; "tf" = tibia and fibula.

# Figure 5

Patelloids in a specimen of the recently extinct thylacine (Supplementary Text S1: Tables S1,S5).

(A-D): *Thylacinus cynocephalus* (UMZC display specimen A6.7-1) with patelloids *in situ*. (A) Photograph of left knee joint in cranial view showing patelloid. (B) Oblique left side view of left and right hindlimbs showing bones and patelloids. (C,D) Slices of left (C) and right (D) knees from micro-CT scans. Note lower density of QF tendon and patelloid (greyer shades) vs. bony tissue (whiter shades) in C and D. Green arrows point to location of patelloid where visible. Red vertical scale bars in C and D are 20mm. Labels: fem = femur; tf= tibia/fibula. Artefacts (bright white lines through some bones) were caused by ferrous wire.

# Figure 6

Ossified patellae in extant marsupials (Supplementary Text S1: Tables S1,S5).

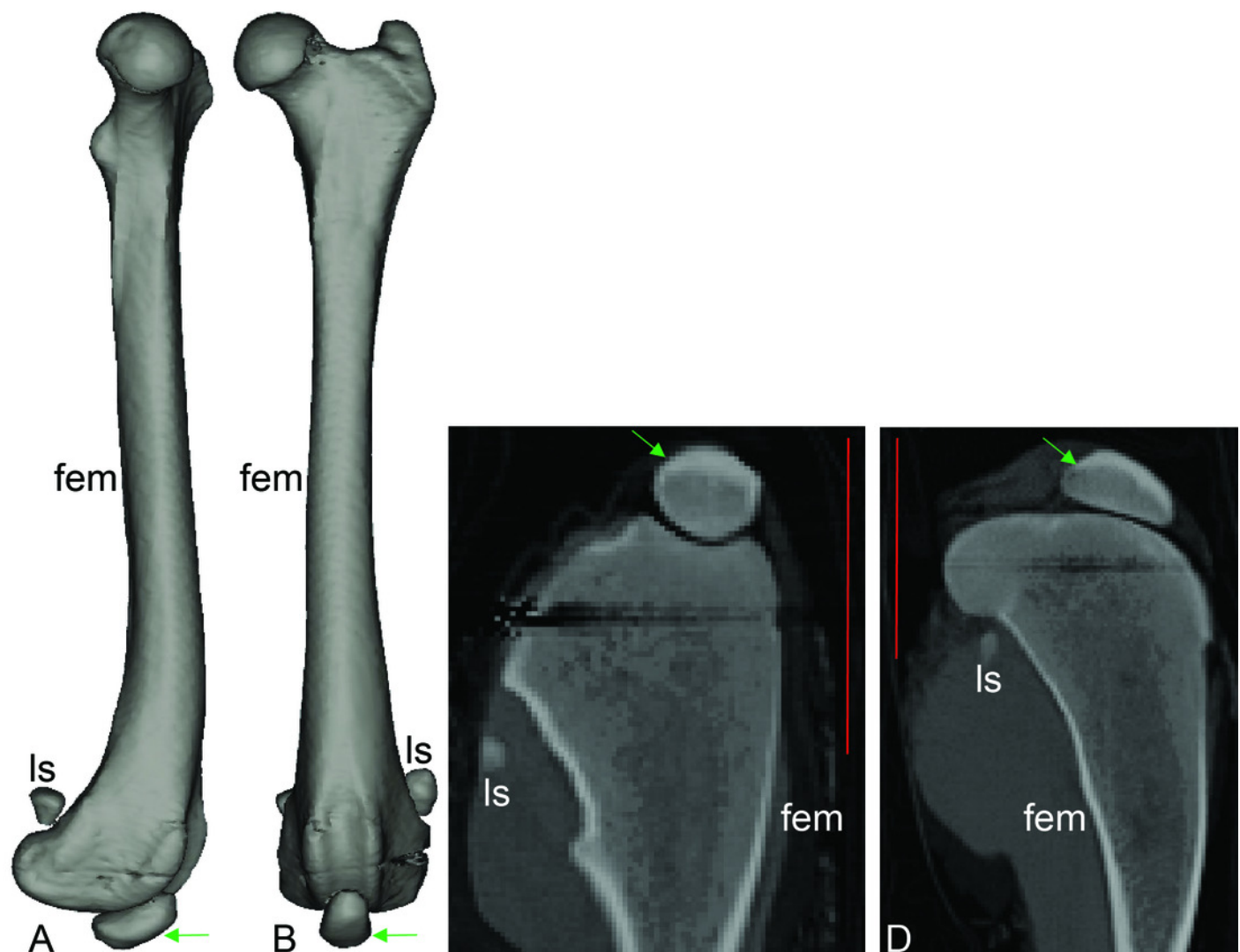
(A) photograph of *Macrotis lagotis* (UMZC specimen A7.1-1) bilby, left (top) and right (bottom) hindlimbs. (B) CT scan reconstruction of *Notoryctes* sp. (UMZC specimen, no number) "marsupial mole", left and right hindlimbs. (C-E) Micro-CT scan slices through knee joints. (C,D): left (C) and right (D) sides of *Caenolestes* sp. shrew opossum (UMZC specimen A.8.2/3). (E) right side of *Isoodon obesulus* (UMZC specimen A7.4/5) bandicoot. Green arrows point to location of ossified patella where visible. Note comparably high density of patella vs. other bony tissue (white shades); cf. Figure 3C,E. Labels: "fem" = femur; "ls" = lateral sesamoid; "pes" = pes (hindfoot); "tf" = tibia and fibula. Red scale bars in B-E are, respectively, 12, 2, 2 and 7 mm.

# Figure 7

Example radiographs of skeletal specimens from NHMUK (Supplementary Text S1: Tables S2,S6).

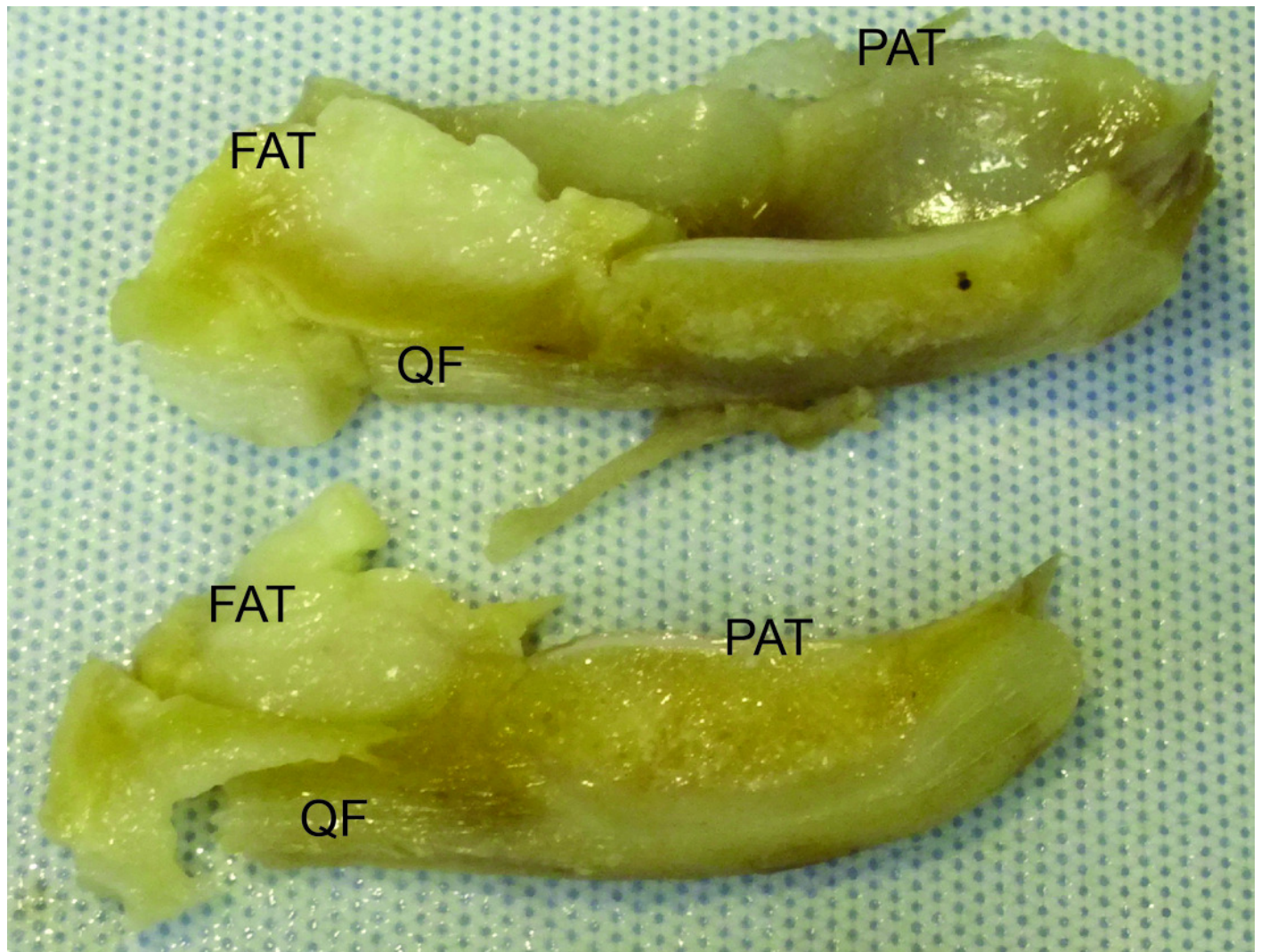
Marker pin has central width of 0.52mm. (A) *Didelphis marsupialis*; specimen NHMUK 1959.11.10.1 (opossum); cranio-caudal view; no patellar ossification. (B) *Vombatus ursinus*; specimen NHMUK 1964.6.29.1 (wombat); medio-lateral view; no patellar ossification.

# Figure 8

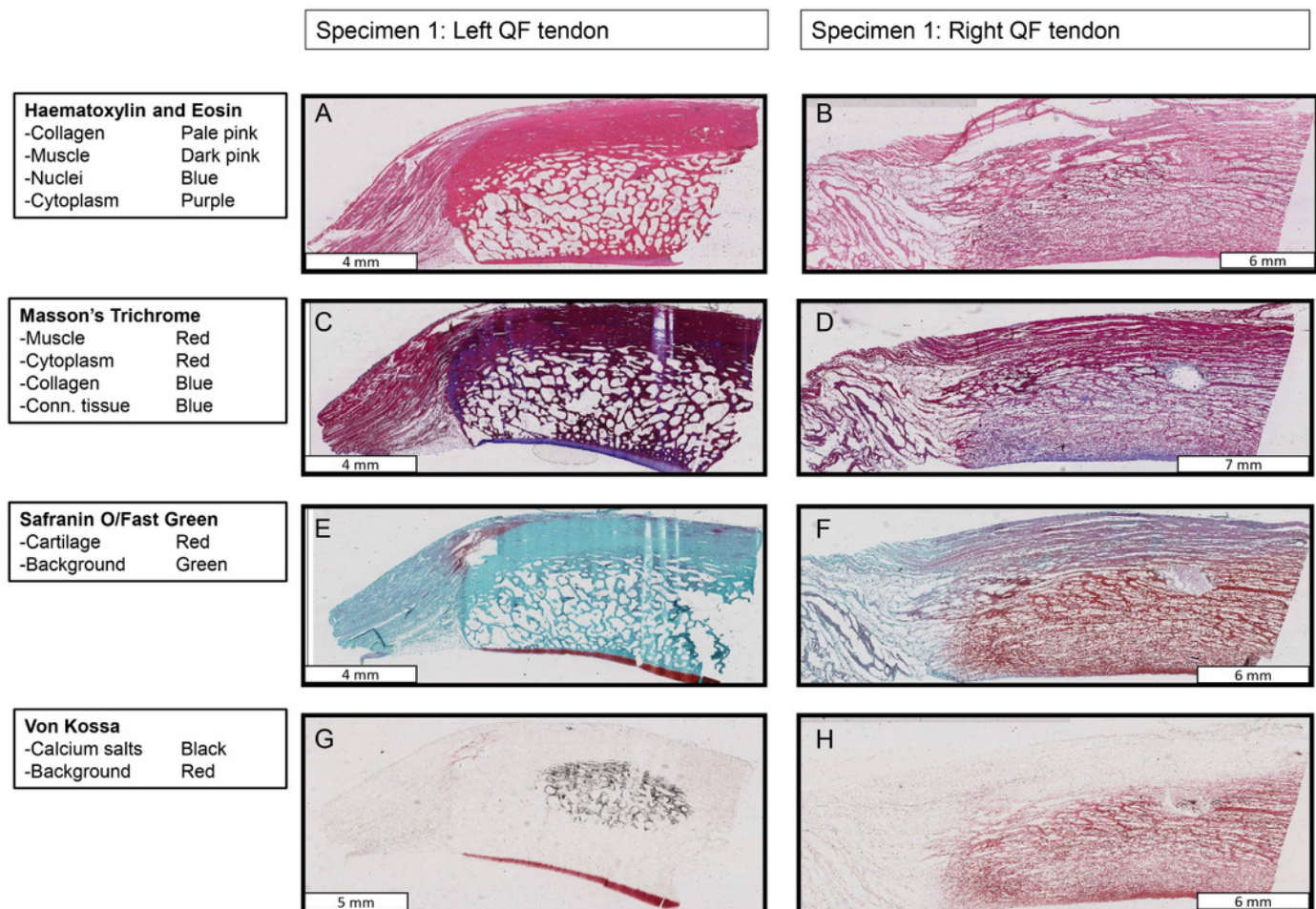
CT scan revealing presence of ossified tissue in the QF tendon of specimen 1: a recently deceased *Macropus rufogriseus* (Supplementary Text S1: Table S3), Bennett's wallaby.

(A, B): 3D reconstructions of femur region from CT scans (bone density only), with medial view in A and cranial view in B; (C, D): longitudinal (C) and mediolateral (D) sections from micro-CT scans. Red vertical scale bars in C and D are 40mm. Green arrow indicates position of ossified patella. Artefacts through femoral epiphyses were caused by ferrous wire.

# Figure 9

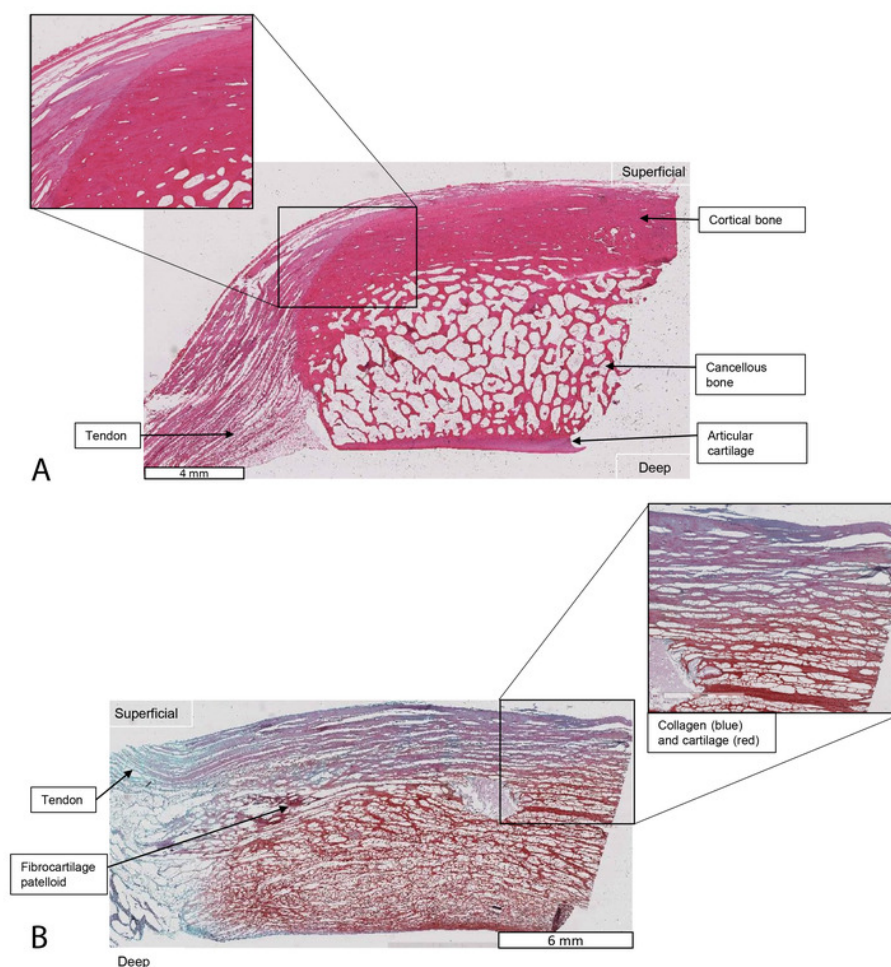
Photograph of the ossified patella, harvested from specimen 1 of *Macropus rufogriseus*, following decalcification and cutting.

Not to scale. Labels: FAT = infrapatellar fat pad; PAT = patella main region; QF = QF tendon.

# Figure 10

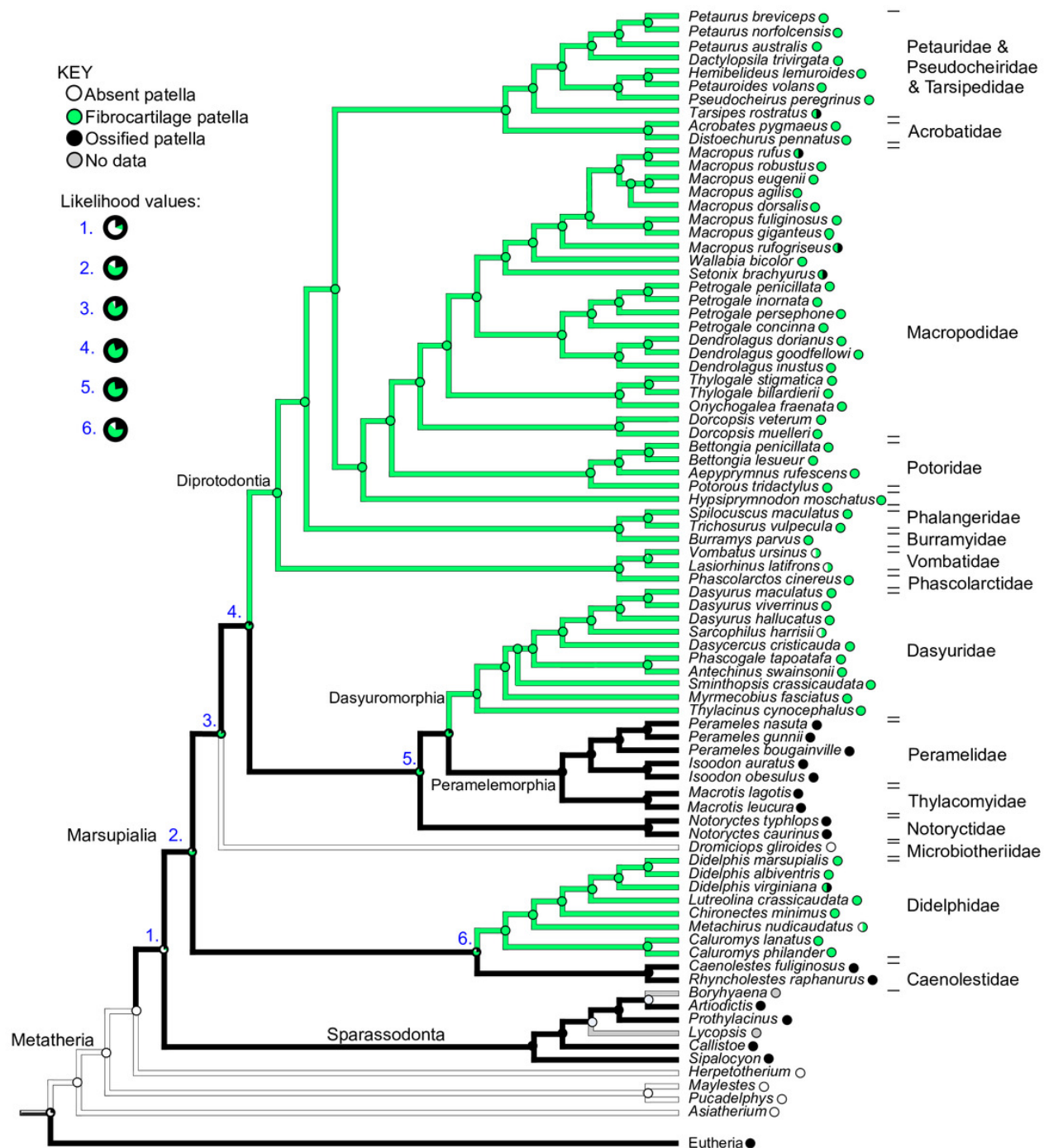
Stained sections from the right and left QF tendons of specimen 1, a *Macropus rufogriseus*.

One section is illustrated for each stain used. Details of tissues highlighted by each stain are indicated on the left of the figure. An ossified patella is demonstrated in the left tendon (A, C, E, G), while a fibrocartilage patelloid is in the right tendon (B, D, F, H).

# Figure 11

Higher-magnification of two stained sections of QF tendons from Figure 10, *Macropus rufogriseus* specimen 1.

Relevant tissues and orientations are indicated. (A): Haematoxylin & Eosin stained section of the ossified patella from the left QF tendon. Inset: detail of tendon-bone junction (enthesis); proximal edge of the patella. (B): Safranin O/Fast Green stained section of the fibrocartilage patelloid from the right QF tendon, in higher-magnification view.

# Figure 12

Ancestral state reconstruction for the patella in marsupial mammals.

Branch colours indicate reconstructed states according to (unordered) maximum parsimony, whilst circles at nodes indicate percentage values of ancestral states according to maximum likelihood (Mk1). Likelihood values for major nodes (#1-6) are magnified beneath the key. Includes only species for which observations were made or literary references found, mapped onto the original tree used. See Results for interpretations.

# Figure 13

Dissections of an adult male koala; right hindlimb in cranial view of knee.

(A) Specimen skinned with superficial surface of patelloid exposed and QF muscle/tendon partially reflected. (B) QF muscle/tendon fully reflected below distal femur to expose deep surface of patelloid. The specimen was obtained by Monash University from Museums Victoria (registered as MUPC5), from the Victorian Department of Sustainability and Environment cull program of 7 March 2014, Bimbi Park, Cape Otway, Victoria, Australia (38 50 02 S, 143 30 47 E) under Flora and Fauna Permit number 10007596. Photos and description courtesy of Hazel Richards. Not to scale. Labels: fem = (distal end of) femur; pat = patelloid; QF = QF muscles/tendon.