The oldest platypus and its bearing on divergence timing of the platypus and echidna clades

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Monotremes have left a poor fossil record, and paleontology has been virtually mute during two decades of discussion about molecular clock estimates of the timing of divergence between the platypus and echidna clades. We describe evidence from high-resolution x-ray computed tomography indicating that *Teinolophos*, an Early Cretaceous fossil from Australia's Flat Rocks locality (121–112.5 Ma), lies within the crown clade Monotremata, as a basal platypus. Strict molecular clock estimates of the divergence between platypus and echidnas range from 17 to 80 Ma, but *Teinolophos* suggests that the two monotreme clades were already distinct in the Early Cretaceous, and that their divergence may predate even the oldest strict molecular estimates by at least 50%. We generated relaxed molecular clock models using three different data sets, but only one yielded a date overlapping with the age of *Teinolophos*. Morphology suggests that *Teinolophos* is a platypus in both phylogenetic and ecological aspects, and tends to contradict the popular view of rapid Cenozoic monotreme diversification. Whereas the monotreme fossil record is still sparse and open to interpretation, the new data are consistent with much slower ecological, morphological, and taxonomic diversification rates for monotremes than in their sister taxon, the therian mammals. This alternative view of a deep geological history for monotremes suggests that rate heterogeneities may have affected mammalian evolution in such a way as to defeat strict molecular clock models and to challenge even relaxed molecular clock models when applied to mammalian history at a deep temporal scale.

Mammalia | Monotremata | phylogeny | molecular clock

The timing of divergence of the two living monotreme clades is of general interest because it bears on basal events in mammalian history and provides independent calibration for understanding temporal aspects of the great radiation of therian mammals. Strict molecular clock estimates of the platypus-echidna divergence time range from 17 Ma to 80 Ma (1–11), with most opinions favoring the younger end of the spectrum (Table 1). Recent discoveries of Mesozoic and earliest Cenozoic fossils hint at a far deeper geological history for monotremes (12, 13); however, these fossils consist mostly of isolated teeth and jaws and their precise relationships are controversial. They are generally relegated to positions at the base of the monotreme stem, and viewed as consistent with a relatively recent divergence between the living platypus and echidna clades.

High-resolution x-ray computed tomography (HRXCT) is enabling a systematic reappraisal of these important and controversial fossils by generating new information on the comparative internal structure of the mandible and dentition (14, 15). In this report, we focus on one such fossil, *Teinolophos trusleri*, and describe new information on derived features of its jaw morphology. We also re-analyzed a large data set of morphological characters relevant to basal mammalian phylogeny, and our results shifted the phylogenetic position of *Teinolophos*, from stem to crown Monotremata. The implications of this seemingly minor adjustment are magnified and amplified by the great antiquity of this fossil. As we report below, this adjustment may broadly affect our understanding of early mammalian history, with special implications for molecular clock estimates of basal divergence times.

Monotremata today comprises five species that form two distinct clades (16). The echidna clade includes one short-beaked species (*Tachyglossus aculeatus*; Australia and surrounding islands) and three long-beaked species (*Zaglossus bruijni*, *Z. bartoni*, and *Z. attenboroughi*, all from New Guinea). The platypus clade includes only *Ornithorhynchus anatinus* (Australia, Tasmania). At first glance, the platypus and echidnas seem as different from each other as they are from therian mammals, yet monotreme monophyly is supported by skeletal morphology (17–22), brain architecture (23, 24), facial electro-receptor arrays of unique structure (25), karyotype (26), and mitochondrial (27, 28) and nuclear gene sequences (29). Monotremes are conventionally recognized as the sister clade to therian mammals, and to retain many plesiomorphic mammalian features that were transformed during therian evolution (30, 31).

Challenges to this conventional view were raised recently, when either an echidna or the platypus was found to be phylogenetically nested within, or as sister taxon to, marsupials. Evidence came from sequence analyses of 18s rRNA (32), and both mitochondrial (9, 32–37) and nuclear genes (7, 38, 39). These findings resurrected the obscure "Marsupionta" hypothesis, which contended that monotremes are derived marsupials who secondarily reacquired such ancient characters as ovipary. Formulated in 1947 by the great morphologist W. K. Gregory (17), this hypothesis has been largely disregarded ever since (29, 31). If true, it would profoundly alter the framework in which mammalian history is understood today.

The Marsupionta hypothesis has been unanimously rejected in favor of the conventional view of monotreme-therian relationships in all recent computed phylogenetic analyses that incorporated fossils and large samples of skeletal characters (18–22, 40–43), including our analysis. The molecular arguments favoring Marsupionta have also been challenged. Some analyses did not simultaneously sample both monotreme clades (9, 32), whereas those that sampled both platypus and echidna clades supported the conventional view of monotreme relationships (11, 27–29, 44). Analyses of large concatenated sets of nuclear

Table 1. Published estimates of echidna–platypus divergence times, including our relaxed molecular clock estimates (bottom three rows)

Date	Data/method
17–25 Ma (10)	Immunoglobulin IgM
18–27 Ma (4)	Molecular clock estimates based on Protamine P1 genes (290 bp for platypus, 311 bp for echidna)
20–45 Ma (6)	Molecular estimates based on mitochondrial ND1 protein sequences and assuming that the echidna-platypus split is 20–30% as old as the monotreme-marsupial split
25 Ma (7)	Molecular clock estimate based on single copy DNA–DNA hybridization data
25–30 Ma (5)	Molecular clock estimates based on partial mitochondrial 12S rRNA gene sequences
28–73 Ma (2)	Molecular clock estimates based on myoglobin, α-globin, and β-globin protein sequences
34 Ma (9)	Molecular clock estimate based on amino acid sequences for 12 mitochondrial proteins
54 Ma (1)	Molecular clock estimate based on α-and β-haemoglobin and myoglobin protein sequences
50–57 Ma (8)	Molecular clock estimate based on α-lactalbumin protein sequences
63.6 Ma (11)	Molecular clock estimate based on 66 nDNA, mDNA, tRNA genes
>63.2 - 61.8 Ma (13)	Fossil (*Monotrematum*), paleomagnetic date
64–80 Ma (3)	Molecular clock estimates based on single copy DNA–DNA hybridization data
63.7 (95.0–39.7) Ma	Relaxed molecular clock estimate based on Woodburne *et al.* IGF2 data (41)
79.5 (110.4–51.6) Ma	Relaxed molecular clock estimate based on van Rheede *et al.* Dataset I DNA data (29)
88.9 (130.8–55.6) Ma	Relaxed molecular clock estimate based on van Rheede *et al.* Dataset I amino acid data (29)

Fig. 1. *Ornithorhynchus anatinus*, in lateral (*A*), dorsal (*C*), and posterior (*E*) views, compared with its extinct relative *Obdurodon dicksoni* (lacking mandible) in lateral (*B*), dorsal (*D*), and posterior (*F*) views. Volumetric reconstructions based on HRXCT, illustrated at same lengths (not to scale). Abbreviations: Den (dentary), Fm (foramen magnum); m1 (sclerified first lower molar); m2 (sclerified second lower molar); Mt (mandibular tubercle); P3 (upper third premolar); P4 (upper fourth premolar); V2 (foramina for maxillary nerve), and V3 (foramen for mandibular nerve).

gene sequences suggest that ribosomal and mitochondrial sequences are most successful in modeling and resolving branching events in relatively shallow time whereas long nuclear sequences preserve stronger signal on the timing of Mesozoic and more ancient events (6, 26, 29, 41, 45). The underlying clock models and calibration rationale for molecular estimates are also matters of debate (5–8, 41).

Owing to the sparseness of crown monotreme fossils, molecular clock models have been the method of choice for most estimates of echidna–platypus divergence timing, and various events in therian history were used as calibration. Fossils still provide decisive evidence on minimum divergence times and are required for molecular clock calibration (46, 47). However, the oldest verifiable echidna fossil is from the middle Miocene (48, 49) and until now, the oldest unequivocal platypus (*Obdurodon*, Fig. 1) dated to the late Oligocene (49–51). These taxa are based on fairly complete skulls whose identities are unmistakable because they possess nearly all of the apomorphies that are so distinctive of their living relatives. None has offered much insight into more distant monotreme history, into what the last common ancestor of the platypus and echidnas looked like, or when it lived. Different events in therian history were used to calibrate the molecular clock models applied to monotreme history, and they produced conflicting timing estimates of the echidna–platypus split (Table 1).

The monotreme stem was long thought to be represented by the extinct Mesozoic taxa Morganucodontidae (Late Triassic–Middle Jurassic) and Multituberculata (Late Jurassic–Paleogene) (52–53). Under that interpretation, the fossil record testified that monotremes and therians had separated by the Late Triassic. However, those relationships were unanimously overturned by computed phylogenetic analyses that incorporated large samples of skeletal characters. Morganucodotids are now thought to be the sister taxon to crown Mammalia, whereas multituberculates are stem therians (18–21, 40–43). The revised phylogeny also altered the evidentiary standing of the Triassic fossils, placing them on the mammalian stem rather than within the crown, and leaving us without any Triassic mammals or any stem monotremes.

Recent discoveries of Mesozoic fossils from the Southern Hemisphere have renewed hope of acquiring genuine clues to early monotreme history. One record is an isolated humerus from the Early Cretaceous ($\approx$106 Ma) Dinosaur Cove locality of Victoria, Australia, that resembles an echidna, but is sufficiently incomplete as to deny unequivocal attribution (54). All of the other fossils consist of isolated teeth and jaws, and the phylogentic placement of each has been challenged on different grounds (40, 41, 55, 56, 60). The Early Cretaceous Australian fossil *Teinolophos* is among these.

A number of researchers have noted that dental morphology allies *Teinolophos* with monotremes (40, 41, 58–60), and specifically with the platypus (31, 56, 61). However, the complete absence of teeth in all known echidnas has left equivocal the nature of dental similarities that *Teinolophos* shares with *Obdurodon* and *Ornithorhynchus*. They could either be indications of true platypus affinities, or plesiomorphic characters present in monotremes ancestrally. Other researchers argued that *Teinolophos* retains a Meckelian canal that held postdentary elements, another plesiomorphic feature placing *Teinolophos* outside of crown monotremes (22, 61). Previous phylogenetic analyses have all favored the hypothesis that *Teinolophos* branched from the monotreme stem (11, 21, 57, 59, 61, 62), in a relationship consistent with a comparatively recent divergence of the platypus and echidna clades.

Also assigned to the monotreme stem by several analyses are *Steropodon*, *Ausktribosphenos*, *Bishops* (Early Cretaceous, Australia), *Ambondro* (Middle Jurassic, Madagascar), and *Asfaltomylos* (Middle–Late Jurassic, Argentina) (57, 58, 62). Believed to represent a clade that includes living monotremes, this

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Fig. 2. *Teinolophos trusleri* (NMV P216575). Volumetric reconstruction of left dentary, from HRXCT, in medial (*A*) and lateral (*B*) views, and in selected coronal cross sections (slice thickness = 0.019 mm). Medial tubercle (Mt) and mandibular canal (V3) are labeled. Complete CT serial section stacks and volumetric animations are available at http://digimorph.org/specimens/Teinolophos_trusleri/216575/.

Fig. 4. Comparative diameters of the mandibular canal (in red) shown in HRXCT coronal slice planes, with the location of the slice marked by a white line on three-dimensional reconstructions of complete skulls in lateral view. (*A*) *Morganucodon sp.* (IVPP 8685), Early Jurassic fossil from China. (*B*) *Ornithorhynchus anatinus* (AMNH 252512), juvenile specimen still in possession of its deciduous dentition. (*C*) *Ornithorhynchus anatinus* (AMNH 200255) adult specimen with keratinous adult dentition. (*D*) *Zaglossus bruijni* (AMNH 157072) adult specimen. (*E*) *Didelphis virginiana* (TMM M-2517) adult specimen. (*F*) *Dasypus novemcinctus* (TMM M-7417), adult specimen. The mandibular canal is labeled (V3). Images not to scale; scaled imagery and complete CT data sets for each are available at www.DigiMorph.org.

assemblage was named ''Australosphenida,'' and this hypothesis of relationships formed the basis for controversial arguments that the tribosphenic molar (57) and middle ear (61, 63) evolved independently in australosphenidans and therians.

For our study, three specimens of *Teinolophos* (specimen numbers: NMV P216750, NMV P216575, and NMV P212933) were scanned at the University of Texas High Resolution x-ray Computed Tomography facility (Figs. 2 and 3). Two were edentulous jaws, and the third was a dentary with three teeth, all from the Flat Rocks locality, Wonthaggi Formation, Bunarong Marine Park, Victoria, Australia, where the type specimen of *Teinolophos* was collected (64). These data were compared with scans of living monotremes including an adult *Ornithorhynchus* and a juvenile still in possession of its deciduous teeth, the fossil platypus *Obdurodon*, the fossil mammaliaform *Morganucodon*, several euticonodont fossils, several multituberculates, and a large collection of extant therian mammals scanned with HRXCT over the last decade (Fig. 4). The new morphological data were used to modify a large morphological data set published by Luo and Wible (21), which we analyzed using parsimony. Insofar as our results were inconsistent with all strict molecular clock estimates for the echidna–platypus split, we applied three different relaxed clock modes using different

sequences, to evaluate whether any molecular clock models is consistent with the interpretation of a deep geological age for the echidna–platypus divergence. Our matrix, modified character scores, sequence analyses, and methodology are described in supporting information (SI) *Text*.

Results

Our analysis found that *Teinolophos* lies within the monotreme crown, as the most ancient member of the platypus clade, Ornithorhynchidae (Fig. 5). Its precise position among ornithorhynchids was sensitive to different taxon samples, but invariably *Teinolophos* clustered with other ornithorhynchids (see *SI Text*). The most compelling evidence to us of its platypus affinities was provided by the HRXCT scans, which revealed the presence of a hypertrophied mandibular canal coursing along the entire length of dentary in a position lateral to the molariform tooth roots, and which exits the ramus medially beneath a large medial tubercle (Figs. 1–3). Among extant mammals, only the platypus has such a hypertrophied canal and medial tubercle (Fig. 4). Whereas diprotodont marsupials have an enlarged canal near the back of the jaw for insertion of the pterygoideus musculature (65), our comparative analysis indicates that only in ornithorhynchids is the mandibular canal hypertrophied along its entire length.

The ornithorhynchid mandibular canal transmits the mandibular artery and hypertrophied mandibular branch of the trigeminal nerve, in support of the electroreceptive bill that gives the duckbilled platypus its common name (66). The bill deploys an array of 60,000 mechanoreceptors along with 40,000 mucous gland electroreceptors. Electroreceptive nerve terminals lie in the ducts of glands that secrete mucous when immersed in water, and they measure electrical profiles of aquatic prey items (25, 67–72). Stimului received by receptors in the bill are transmitted via comparatively huge mandibular and maxillary branches of the trigeminal nerve to an expansive population of neurons in the S1 somatosensory cortex that are bimodally responsive to both mechanical and electrical signals (25, 69, 70). Echidnas also have

Fig. 3. *Teinolophos trusleri* (NMV P216750). Volumetric reconstruction of right dentary, built from HRXCT, in medial (*A*) and increasingly oblique views (*B* and *C*). (*D*–*J*) Selected coronal cross sections (slice thickness = 0.012 mm) of this jaw, with slice sequence positions indicated by numbers and vertical white lines in *A* to show position of the slice plane. Abbreviations: Alv, tooth alveoli; V3, mandibular canal; Mt, Medial tubercle. Complete CT serial section stacks and volumetric animations are available at http://digimorph.org/specimens/Teinolophos_trusleri/216750/.

Fig. 5. Cladogram showing relationships of *Teinolophos* to other mammals and their extinct relatives among Cynodontia (see *SI Text* for matrix and details of methodology).

actual bone and teeth dissolved. However, the dental resemblances to *Teinolophos* and *Obdurodon* are thoroughly documented (40, 41, 59, 60), and it preserves the edge of a large mandibular canal. The Paleocene fossil *Monotrematum* (74), based on three teeth from Argentina, is probably also a member of the platypus clade (Ornithorhynchidae) (31).

Optical data (61) has been interpreted as evidence for the presence of postdentary bones in *Teinolophos*. However, HRXCT data show no evidence of a postdentary trough or postdentary bones, suggesting that *Teinolophos* had a "standard" mammalian middle ear in which the auditory ossicles were separate from the lower jaw and hung suspended beneath the otic capsule, as in the platypus today (75, 76). This finding adds to the mounting evidence (60) that "Australosphenida" is a polyphyletic assemblage, with several of its members (*Teinolophos*, *Steropodon*) belonging to crown monotremes, whereas others (*Ausktribosphenos*, *Bishops*, *Ambondro*, *Asfaltomylos*) clustered consistently with therians. The precise positions of the latter taxa were also sensitive to taxon sampling in our analyses, but invariably they clustered with therian mammals (see *SI Text*). Should the Australosphenida hypothesis fail, then so too would assertions based thereupon that the mammalian middle ear and tribosphenic molars evolved convergently (57, 58, 61, 62, 77).

The Flat Rocks locality was dated by using the fission track method at 121–112.5 Ma (64). The finding that *Teinolophos* is a platypus indicates that the platypus and echidna clades diverged during or before the Early Cretaceous. This date is more ancient by a factor of 7 than the youngest, and 50% older than the oldest strict molecular clock estimates (Table 1). The recent characterization of monotreme history as a "long-fuse" clade, whose diversification into platypus and echidna clades postdated the Cretaceous–Tertiary boundary (11), is difficult to reconcile with our more ancient divergence estimate, nor is there evidence of a diversity "explosion" at any time in monotreme history.

Although far older than any previous estimate, the accumulation of anatomical novelty in even the oldest monotreme fossils suggests that our estimate may underrepresent the actual timing of the split between platypus and echidna. Viewing *Teinolophos* and *Steropodon* as platypuses in their ecological aspect suggests that, since the Early Cretaceous, rates of ecological exploitation and morphological diversification among monotremes may have been far slower than is the case in most or all therian clades. The 1,000-fold difference in species diversity found today (5 monotremes vs. 5,362 therian species; ref. 16) may be another indication that monotremes evolved at far slower rates than therians. Several molecular clock studies (6–9) have also suggested the possibility of a molecular evolution rate slowdown in monotremes. A rate slowdown in monotremes will result in estimates for the platypus–echidna divergence that are too young if calibrations are derived from therian taxa with faster rates of molecular evolution.

Given differences in rates of molecular evolution, we applied a relaxed molecular clock method (refs. 78–80; see *SI Text*) to reanalyze both the DNA and amino acid sequence versions of van Rheede *et al.*'s (29) data set I for 21 mammalian taxa. Each analysis gave a different estimate. The amino acid data of van Rheede *et al.*'s (29) data set I yielded a point estimate of 88.9 Ma for the platypus–echidna split, with 95% credibility intervals ranging from 130.8 to 55.6 Ma. This result overlaps broadly with the 121–112.5 Ma date for *Teinolophos*. However, the DNA data set of van Rheede *et al.* yielded a point estimate of 79.5 Ma (credibility interval 110.4 to 51.6 Ma). Previously, Woodburne *et al.* (41) reported a relaxed clock point estimate of 63.7 Ma (credibility interval 95.0–39.7 Ma) for the platypus–echidna split based on IGF2 amino acid sequences.

Discussion

Even considering *Teinolophos* as a crown montreme, the monotreme fossil record remains dismally sparse and open to

electroreceptive capabilities in their beaks (72), but their sensitivity is far less than in the platypus bill. The long-beaked echidnas have only ≈2,000 electroreceptors, whereas the short-beaked echidna has only ≈400 (25), which they use in probing moist substrate for food. Both have comparatively narrow mandibular canals, reflecting the plesiomorphic condition that is found in *Morganucodon* and all therians sampled (Fig. 4). Electroreception therefore appears to be an apomorphic characteristic of Monotremata, whereas the evolution of a specialized duckbill for high-resolution aquatic electroreception is unique to the platypus clade. *Teinolophos* preserves the oldest evidence of a duckbill in its hypertrophied mandibular canal.

Our analysis also affirms that the slightly younger (110 Ma) Australian fossil *Steropodon* (12, 73), known from a single broken jaw, is also a platypus. This unique specimen was preserved as an opal infilling of a natural mold, left after the

interpretation. Our results are consistent with estimates of a Triassic date for the monotreme–therian split (41) (*SI Text*), although as yet we have no Triassic crown-mammalian fossils that would offer direct corroboration. If the new position of *Teinolophos* is upheld, crown monotremes had originated and the platypus and echidna clades were established by the Early Cretaceous.

Monotremes have left only a meager fossil record, but what is known at present is consistent with the view that soon after their divergence, in or before the Early Cretaceous, monotremes settled into rates of molecular and morphological evolution and speciation far slower than in the living clades of therian mammals. Even the monotreme metabolic rates and ventilation rates are much slower than in therian mammals of similar body mass, and their body temperature is lower as well (81). In what measure and to what degree these various rates are coupled or were independently evolving phenomena remains to be determined. It is also difficult to discern in which respects monontremes are simply expressing plesiomorphic mammalian rates, whether there have been apomorphic slowdowns in monotremes that evolved following their divergence from therians, or to what degree therian history can be characterized by rate accelerations over the ancestral states for mammals. In any case, our results suggest that different mammalian clades were subject to evolutionary rate heterogeneities that are incompatible with strict molecular clocks and difficult to accommodate even when relaxed molecular clock models are applied to mammalian history on a deep temporal scale.

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