Insight into the growth pattern and bone fusion of basal birds from an Early Cretaceous enantiornithine bird

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Bird skeletons exhibit remarkable modifications that allow for flight. The most distinguishable features are the fusion of the bones in the hand, feet, and pelvis into composite rigid and bony structures. However, the historical origins of these avian bone fusions remain elusive because of the rarity of transitional fossils and developmental studies on modern birds. Here, we describe an Early Cretaceous bird (120 Mya) that has fully fused alular-major metacarpals and pelvis. We discuss the manus and pelvis fusions across Paravian phylogeny and demonstrate that these features evolved independently across nonavian theropods, Enantiornithes, and Ornithuromorpha. The fusions of these bones are rare in known nonavian theropods and Early Cretaceous birds but are well established among Late Cretaceous and modern birds, revealing a complicated evolution pattern unrecognized previously. We posit that the developments of bone fusion were polymorphic close to the origin of birds, resulting in the varying degrees of fusion in Paraves. However, that development polymorphism appears to be fundamentally restricted along the line to modern birds by the Late Cretaceous, where all birds have a completely fused manus and pelvis. Such changes likely correspond to a refinement of flight capability. Alternatively, the degree of bone fusion in this primitive bird may have been related to modifications in genes or developmental paths. Future studies and fossil discoveries are required to clarify these hypotheses and pinpoint the developmental pathways involving the bone fusions in early avian evolution through to their modern pattern.

bone fusion | bird | Cretaceous | histology | development

Enantiornithes, arguably the most diverse clade of Mesozoic birds, have been reported from every continent except Antarctica (1, 2). More than half of the known global diversity of the Enantiornithes were recovered from the Early Cretaceous Jehol Biota, northeastern China (1), with the bird-bearing horizons spanning from 120 to 131 Mya (3, 4). The numerous complete and articulated Jehol enantiornithines, some of which preserve feathers, stomach contents, and even traces of soft tissues (5, 6), have significantly increased our understanding about the early history and biology of this avian group. Here, we describe an enantiornithine specimen (Fig. 1 *A* and *B*), referrable to *Pterygornis*, from this biota. The holotype and previously only known specimen of *Pterygornis* is incomplete, particularly lacking the cranial and pelvic bones (7), but those are exquisitely preserved in this referred specimen, allowing us to further reconstruct the skeletal morphology for this taxon and determine its phylogenetic position. We performed osteohistological analysis on the referred specimen using a thin section of the long bones and suggests that *Pterygornis* could reach skeletal maturity in approximately 1 y, a growth strategy that most living birds exhibit (8). In contrast, the growth rate is much slower in most other enantiornithines, and it took them several years to reach adulthood (9). *Pterygornis* shows fully ankylosed alular-major metacarpals and pelvis, distinguishable from most known Early

Cretaceous birds. The developmental mechanism underpinning such rare bone fusion in early birds remains largely unexplored.

Results

Description and Comparison. The specimen, Institute of Vertebrate Paleontology and Paleoanthropology (IVPP) V16363 (Table S1), can be referred to as the enantiornithine *Pterygornis dapingfangensis* in having the following diagnostic characteristics of this taxon: The lateral margin of the coracoid is strongly convex, making the proximal end of the coracoid medially curved; the sternum has an external rostral spine and a pair of craniolateral processes; and the lateral trabeculae of the sternum are robust, bearing large fan-shaped caudal expansions that extend as far caudally as the xiphoid process (7). Descriptions of the skull, forelimb, and the pelvic girdle are presented in the main text, and other body regions are provided in *SI Text*.

The frontal processes of the premaxillae project caudodorsally, forming a 19° angle with the maxillary processes (Fig. 2 *A* and *B*). The most striking feature is that the premaxillary corpus is pierced by a foramen on the cranioventral corner of the external naris (Fig. 2*B*). The foramen is oval in shape with the long axis craniocaudally oriented, and it is larger than the crown of the premaxillary teeth, precluding it from simply being a nutrient foramen. In addition, the smooth margin of the foramen weakens the possibility of this being a preservational artifact. No comparable structure, to our knowledge, has been reported in stem or crown birds (10, 11), or theropod dinosaurs (12). Therefore, it is considered as an autapomorphy here. The premaxilla has five teeth (Fig. 2*B*), but most other enantiornithines bear four (4). The jugal process of the maxilla extends caudal to the ventral process of the lacrimal, contributing to the

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Significance

We report an Early Cretaceous bird from 120 My ago that has a completely fused carpometacarpus and pelvis, pushing back the date for these avian traits by over 40 My. We suggest that this taxon grew more rapidly than other basal birds, but the degree of bone fusion is not causally linked with growth pattern in primitive birds. We hypothesize that the surprisingly high degree of bone fusion in basal birds may have been environmentally induced pertinent to flight or alternatively resultant from genetic modifications. Future developmental studies focusing on the development of bone fusion are needed to test these hypotheses and illustrate how the skeletal system of living birds achieves its modern shape.
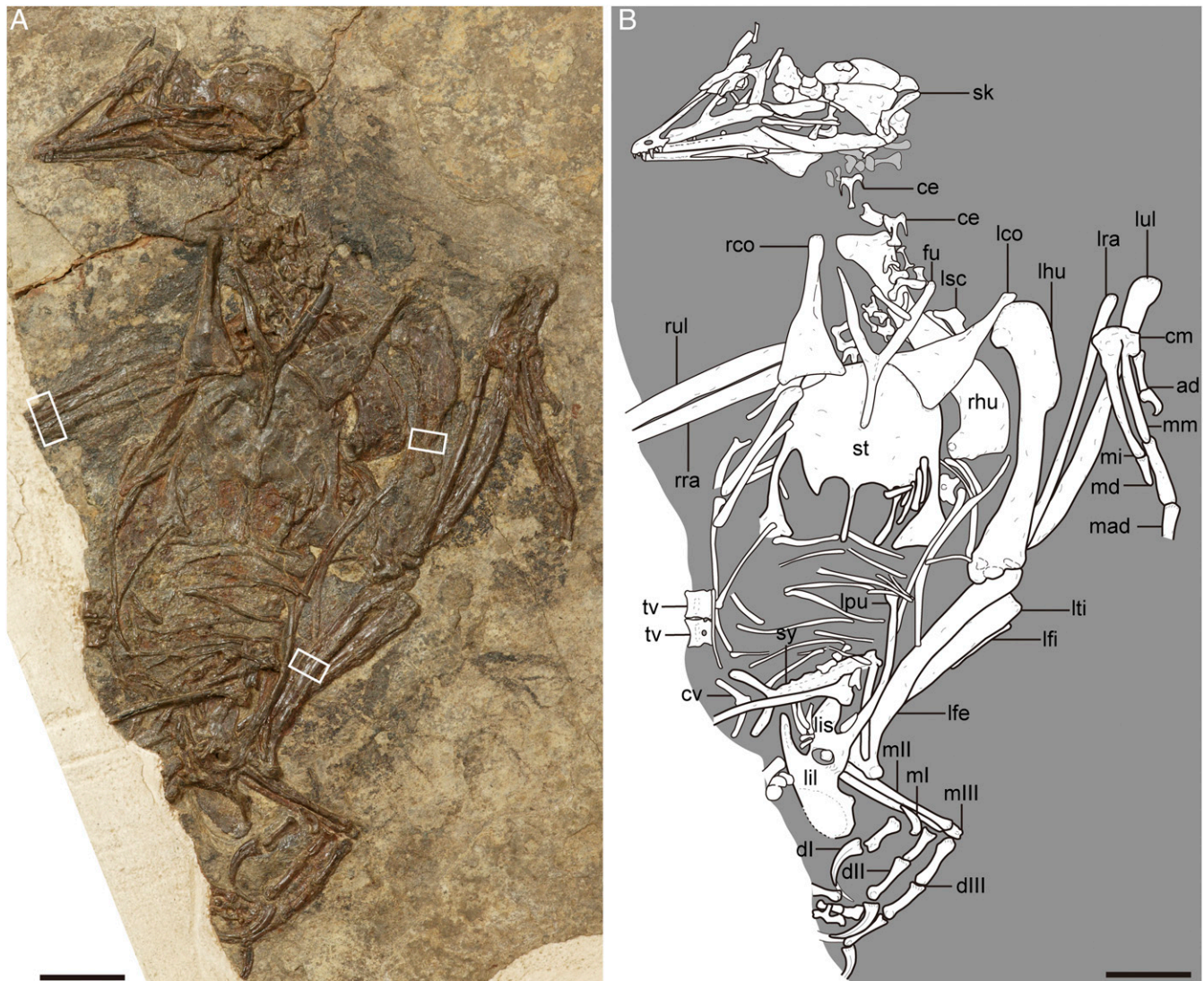

Fig. 1. Photograph (*A*) and interpretative line drawing (*B*) of the referred specimen of *P. dapingfangensis*, IVPP V16363. The white boxes in *A* denote the positions of the bone samples used in the histological analyses. ad, alular digit; ce, cervical vertebra; cm, carpometacarpus; cv, caudal vertebra; dI–III, digit I–III; fu, furcula; lco, left coracoid; lfe, left femur; lfi, left fibula; lhu, left humerus; lil, left ilium; lis, left ischium; lpu, left pubis; lra, left radius; lsc, left scapula; lti, left tibiotarsus; lul, left ulna; mI–III, metatarsal I–III; mad, major digit; md, minor digit; mi, minor metacarpal; mm, major metacarpal; rco, right coracoid; rhu, right humerus; rra, right radius; rul, right ulna; sk, skull; st, sternum; sy, synsacrum; tv, thoracic vertebra. (Scale bar, 10 mm.)

cranioventral corner of the orbital. At least three maxillary teeth are present on each side. The lacrimal is T-shaped, and its ventral process contacts the maxilla medially. The parietals are unfused to each other medially. The otic process of the quadrate is not differentiated into the squamosal and prootic captitula. A mandibular symphysis is absent. The meckelian groove is not completely covered by the splenial. The groove is deep and terminates just short of the rostral end of the dentary. The surangular bears a well-developed retroarticular process.

The bicipital crest of the humerus bears a distinct pit-shaped fossa on its craniodistal surface (Fig. 2*C* and Fig. S1*B*). Previous studies hypothesized that the fossa provided the attachment for an unknown muscle (2). Wang et al. (13) further argued that it served as the insertion site of the scapulohumeralis muscle, which originates from the lateral surface of the scapula and is responsible for the retraction of the humerus (14). The dorsal condyle is elliptical and strongly inclined dorsally. The ventral condyle is nearly transversely oriented and projects less cranially than the dorsal condyle. Above the dorsoventral border of the

ventral condyle, there is a large process that projects as cranially as the dorsal condyle (Fig. 2*D* and Fig. S1 *A* and *B*). This process is probably homologous to the ventral supracondylar tubercle of modern birds (10). No similar structure is observed in other enantiornithines, basal ornithuromorphs, and more primitive birds (2, 15, 16). In modern birds, the ventral supracondylar tubercle provides the attachment for the ventral collateral ligament of the elbow joint (17). The ulna is bowed proximally and straight distally, and it is more robust than the radius (Fig. 2*C*). The alular metacarpal is completely fused with the major metacarpal throughout its length (Fig. 2 *C* and *D*). In contrast, these two bones are at best only proximally fused in other Early Cretaceous birds (7, 16, 18). As in other enantiornithines, the minor metacarpal is only fused with the major metacarpal proximally, and it extends further distally than the major metacarpal. The alular digit is reduced, terminating proximal to the distal end of the major metacarpal. The minor digit only preserves one phalanx, and the manual phalangeal formula is 2–3–1.

EVOLUTION

Fig. 2. Detailed morphologies of *P. dapingfangensis* showing the fused manus and pelvis. (*A* and *B*) Skull in left lateral view of IVPP V16363. (*C*) Left forelimb of IVPP V16363. (*D*) Left hand of IVPP V20729 in dorsal view. (*E* and *F*) Pelvis and hindlimb of IVPP V16363. ac, acetabulum; ad, alular digit; alm, alular metacarpal; at, antitrochanter; cv, caudal vertebra; dI–III, digit I–III; de, dentary; en, external nasal; fe, femur; fh, femoral head; fi, fibula; fr, frontal; hu, humerus; hy, hyoid; il, ilium; is, ischium; ju, jugal; la, lacrimal; ma, maxilla; mad, major digit; mam, major metacarpal; mg, meckelian groove; mid, minor digit; mim, minor metacarpal; mtI–III, metatarsal I–III; na, nasal; pa, parapophysis; pm, premaxilla; pos, postacetabular wing of ilium; pr, parietal; pre, preacetabular wing of ilium; pu, pubis; qu, quadrate; ra, radius; rep, retroarticular process; sp, splenial; su, surangular; sy, synsacrum; ti, tibiotarsus; tv, thoracic vertebra; ul, ulna; vs.t, ventral supracondylar tubercle. The arrow indicates the presences of a foramen on the premaxilla. The five arrowheads denote the five premaxillary teeth. [Scale bar, 10 mm (*A–C*, *E*, and *F*) and 5 mm (*D*).]

The pelvic bones, absent in the holotype, are well preserved in IVPP V16363 (Fig. 2 *E* and *F*). Surprisingly, the ilium, ischium, and pubis are completely fused with one another, a feature absent in all of the other Early Cretaceous enantiornithines, with the exceptions of *Qiliania* and *Piscivorenantiornis* (19), and that absence occurs in many specimens that are widely regarded as fully adult (1, 4, 13). Therefore, the absence of fusion cannot be attributed exclusively to ontogenetic variations given so many examples. An antitrochanter is weakly developed on the

caudodorsal corner of the acetabulum. The pubes are caudally directed, forming a 57° angle with the long axis of the ilium that is larger than in some enantiornithines—for example, *Qiliania* (28°) and *Linyiornis* (37°). The distal end of the pubis flares into a pubic boot. The lateral surface of the ischium is essentially flat. We performed comprehensive phylogenetic analyses using combined information from both the holotype and IVPP V16363, and *Pterygornis* is recovered in a derived position within the Enantiornithes (*SI Text* and Fig. S2).

Bone Histological Description. The humeral cross-section is crushed (Figs. S3*A* and S4 *A* and *B*). The cortex comprises parallel-lamellar bone tissue with predominantly longitudinal-oriented canals, although oblique canals are observed. A thin layer of endosteal-derived lamellar bone (the inner circumferential layer, ICL) occurs, riming the medullary cavity (Fig. S3*A*). The osteocyte lacunae embedded in the bone matrix external to the ICL are flattened and well organized in parallel to the external margin, and their density decreases toward the periosteum (Figs. S3*A* and S4*A*). In some areas of the section, the osteocyte lacunae are globular and randomly dispersed (Fig. S4*B*). Where the periosteal margin of the bone wall is intact, a distinct line of arrested growth (LAG) is present and marks the onset of the deposition of lamellar bone with transversely oriented collagen fibers, designating the presence of an outer circumferential layer (OCL; Fig. S3*A*). The OCL is avascular and contains flattened osteocyte lacunae. No secondary osteons are visible (see *SI Text* for histological description of the ulna and radius).

The femoral cortex is stratified into three layers, with a thick middle layer bounded internally by the ICL and externally by the OCL (Fig. S5). The ICL is relatively thick, measuring more than one quarter of the thickness of the preserved bone wall (Figs. S3*C* and S5). However, this proportion should be treated with caution because the external margin has been worn off to some extent. The ICL is delimited from the external bone tissue by a reversal line as in the radius (Fig. S3*D*). The middle layer is composed of parallel-lamellar bone tissue with longitudinally and reticular-oriented canals (Fig. S5*B*), although some areas exhibit fibro-lamellar bone tissue. Secondary osteons are developed. Serendipitously, the sampled cross-section happens to record the phase when a secondary osteon interrupts the reversal line (Fig. S3*D*). The osteocyte lacunae in the middle layer are flattened and organized parallel to the external margin, and this is most pronounced in areas close to the ICL and OCL. However, in the locally restricted fiber-lamellar bone tissues, the osteocyte lacunae are plump and randomly distributed (Fig. S5*B*).

Discussion

Compared with other enantiornithines and basal birds, the most striking feature of *Pterygornis* is the high degree of skeletal fusion, particularly the manus and pelvis (Fig. 2 *C–F*). The alular and major metacarpals are fused throughout their length, as in crown birds (Fig. 2 *C* and *D*). The alular metacarpal provides the attachment for the M. extensor carpi radialis that originates from the dorsal epicondyle of the humerus (10). The wing can be extended through contraction of that muscle (10), and during that extension, the alular metacarpal undergoes considerable tension. Without fusion to the major metacarpal, the tension on the alular metacarpal can only be transferred to the rest of the manus by means of connective tissue between the bones, which is energetically costly and the alular metacarpal (and manus) could be damaged if the instantaneous force increases beyond a certain point. Instead, by being fused to the carpometacarpus, the alular metacarpal should be stabilized significantly more efficiently in control of the bastard wing, and the tension produced by muscle contraction would be transferred across the whole hand as a unit. The fusion between the alular and major metacarpals are absent in nonornithothoracine birds, including Confuciusornithidae,
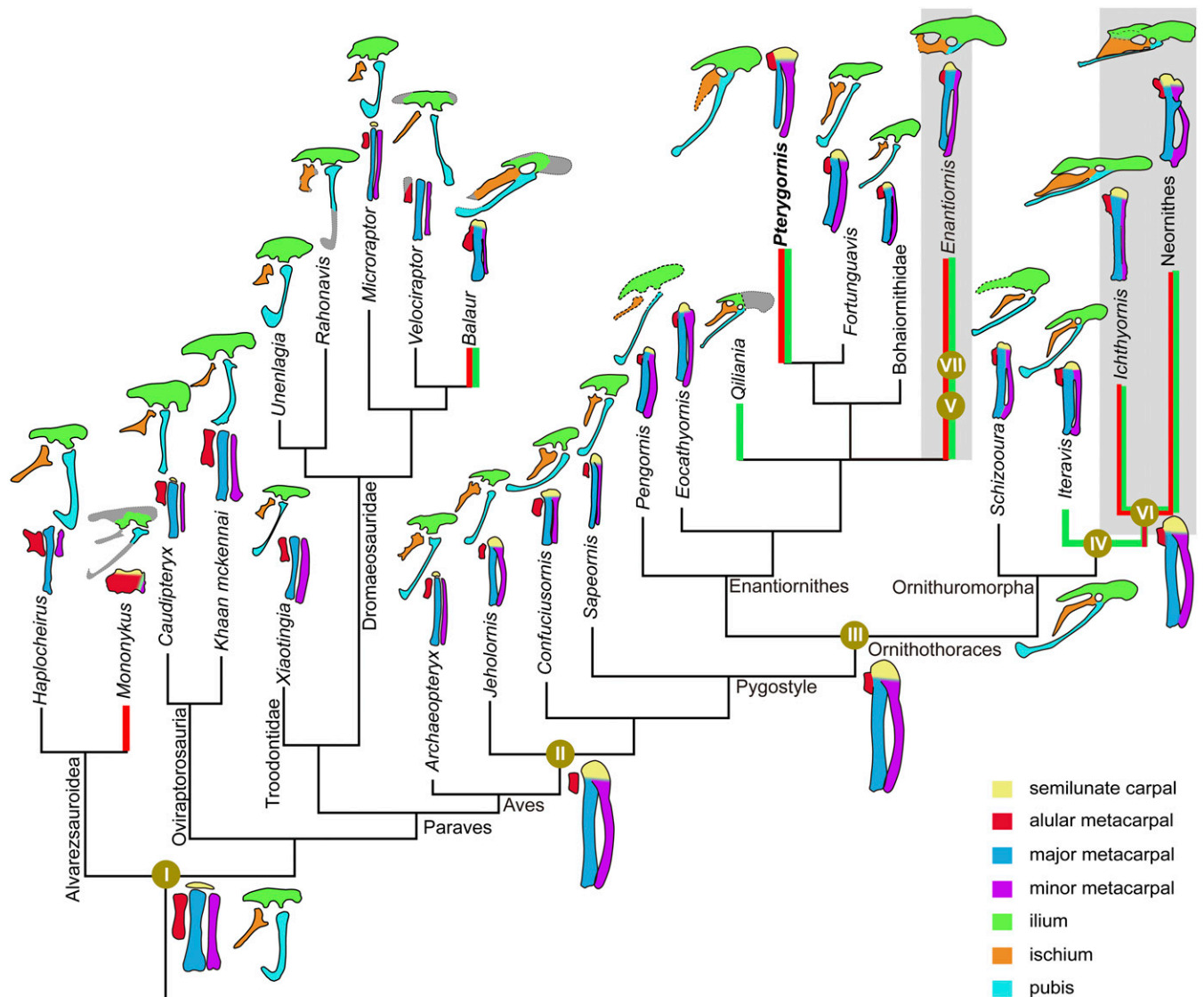
Fig. 3. Paravian phylogeny showing the major changes of manus and pelvis fusions. The ancestral conditions of the pelvis and manus fusions of the major nodes were reconstructed using the parsimonious method in Mesquite. Major changes are summarized below: I, the metacarpals and pelvis unfused in adults; II, the semilunate carpal fused with the proximal ends of the major and minor metacarpals, with the alular metacarpals separated; III, the alular metacarpal fused with the major metacarpal proximally but separated distally; IV and V, the ilium, ischium, and pubis fused around the acetabulum; VI and VII, alular and major metacarpals completely fused along their length. The green thick lines denote the taxa with fused pelvis, and the red thick lines indicate taxa with fused alular and major metacarpals. Late Cretaceous birds are denoted in shaded background. The line drawings are not to scale.

Sapeornithidae, and *Archaeopteryx* (refs. 15, 18, and 20 and Fig. 3 and Fig. S6). In more derived Early Cretaceous birds, Enantiornithes and Ornithuromorpha, the alular and the major metacarpals are separated distally, while ankylosed proximally (refs. 1, 11, and 21 and Fig. 3 and Fig. S6). In contrast, these two bones are completely fused to one another in both clades in the Late Cretaceous (2, 22). Fusion of the alular and major metacarpals is rare among nonavian theropods, with some exceptions such as derived alvarezsauroids, the enigmatic *Avimimus*, and the dromaeosaurid *Balaur* (Fig. 3). However, the manual morphology of those nonavian theropods differs substantially from the avian hands and did not function in flight. For instance, the unique manus in alvarezsauroids has been regarded as adaptation for digging (23). We performed a parsimony-based ancestral state reconstruction for the alula-major metacarpals and pelvis fusions across the phylogeny of Paraves. The result indicates that the fusion of the alular and major metacarpals evolved

convergently in these nonavian dinosaurs, Enantiornithes and Ornithuromorpha (Fig. 3 and Fig. S6 and SI Text).

Before the discovery of IVPP V16363, the three pelvic bones remain unfused in ontogeny in nearly all known Early Cretaceous birds with the exceptions of two enantiornithines, *Qiliania* and *Piscivorenantiornis*, and some ornithuromorphs such as *Gansus* (Fig. 3 and Fig. S6 and SI Text). The pelvic fusion is similar among these taxa, the three pelvic elements ankylosed around the acetabulum. In most modern birds, the ischium and ilium are fused caudally to enclose the caudal margin of the ilioischiadic foramen (10, 24), but this feature is absent in all of the Mesozoic birds. In living birds, the pelvis incorporates individual elements to form an immobile, weight-bearing structure, withstanding strain exerted by hindlimb muscles during locomotion (25). A fused pelvic girdle is occasionally present in some nonparavian theropods, including some ornithomimids, coelophysoids, and ceratosaurs (26). The pelvic girdle is unfused in Oviraptorosauria

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(27), the immediate outgroup to Paraves. Within Paraves, pelvic fusion is only known in several derived dromaeosaurids such as *Balaur* and *Hesperonychus* (27). In contrast, these elements remain separated in basal dromaeosaurids, including *Mahakala*, *Rahonavis*, and *Unenlagia* (27), the known troodontids, and basal Aves (Fig. 3). The ancestor state reconstruction revealed the unfused pelvis as the ancestor condition of Paraves, and the pelvic fusion evolved independently and on multiple occasions in lineages of Dromaeosauridae and Aves (Fig. 3 and Fig. S6).

Embryonic development of living birds has attracted considerable attention recently, and bone ossification sequences for many species are well established (28). Unfortunately, much less attention has been paid to how the compound bones become fused to one another, because much of bone fusion occurs after hatching, and most developmental studies focus only on the embryonic stages. In many living birds, the pelvic fusion completes long before the age of 1 y—for example, around 140 d posthatching in domestic fowls (for carpometacarpus, around 120 d of posthatching; ref. 29). However, osteohistological studies show that the pelvis remains unfused in basal birds that are more than 2 y old—for example, *Jeholornis*, *Confuciusornis*, *Sapeornis*, and Enantiornithes (30–32). A fused pelvis is common among Late Cretaceous Enantiornithes and Ornithuromorpha (22, 33). It appears that the unfused pelvis in adult individuals of Early Cretaceous birds is plesiomorphically inherited from their dinosaurian ancestors and that pelvic fusion evolved convergently in late history of the Enantiornithes and Ornithuromorpha. Early Cretaceous ornithuromorphs generally exhibited a relatively higher degree of bone fusion than contemporaneous enantiornithines (21). For example, the tarsometatarsus is unfused distally in enantiornithines (1, 2) but completely fused in most ornithuromorphs except the most basal ornithuromorph *Archaeorhynchus* (34). Interestingly, osteohistological studies suggest a similar growth pattern for *Archaeorhynchus* and enantiornithines that grew much slower than most other ornithuromorphs (34).

Bone microstructure of *Pterygornis* indicates that its growth rate had slowed considerably before its death, demonstrated by the presence of the ICL, OCL, secondary osteons, and the predominant parallel-lamellar bone tissue (Figs. S3–S5). All of these features generally are accepted to signal the cessation of active growth (9, 35, 36). Enantiornithes typically grew slowly and underwent several instances of growth stoppages before reaching adulthood, a growth pattern reflected in their bone histology where the cortex is mainly formed by parallel-lamellar or lamellar bone tissue containing multiple LAGs in the deep cortex (9, 30). In contrast, *Pterygornis* only have one LAG, and it is positioned close to the periosteal margin, suggesting that skeletal maturity was reached within 1 y with minimal additional later growth. The growth pattern of *Pterygornis* is distinct from most other enantiornithines but similar to *Cruralispennia* and *Confuciusornis* (31, 37). With all of these observations, one would argue that the manus and pelvis fusions in *Pterygornis* stem from their rapid growth. This assumption is questionable, because the pelvis is unfused in *Confuciusornis*, *Cruralispennia*, and basal ornithuromorphs that grew relatively fast, but a fused pelvis is present in Late Cretaceous enantiornithines that grew much slower than *Pterygornis* (9). Therefore, we posit that the degree of bone fusion is not closely related with the growth strategy in basal birds.

Although rare, some nonavian coelurosaurian theropods exhibit varying degrees of fusion in the manus and pelvis (27) and probably evolved convergently (earlier in this section). Nonavian dinosaurs had the potential to fuse these bones, but normally they did not. These bones show varying degrees of fusion in Early Cretaceous birds but otherwise are completely fused in adults of Late Cretaceous species. In a Darwinian model of evolution, the origin of a character will not be step-wise but rather be accompanied by varied states resulting from the developmental polymorphism related to that character (38). The varying degrees of fusion (here, in the alular-major metacarpals and pelvis) in theropod dinosaurs and birds indicate that the developmental trajectories in charge of bone fusion are polymorphic. The fact that all birds across clades have a fused hand and pelvis by the Late Cretaceous indicates that such developmental polymorphism was fundamentally "pruned" and that ontogenetic bone fusion became fixed in avian evolution. So what mechanism led to selection in favor of increased fusion among the manus and pelvis to such an extent that it occurs exclusively in later birds and resulted in the loss of other developmental polymorphisms along the line to crown birds, making bone fusion become "locked in" across avian phylogeny?

Biological novelties can stem from the emergence of new genes or regulatory systems, or a combination of both (39, 40). Great progress has been made regarding the phylogeny of crown birds recently from genomic analyses (41), but pinpointing the specific genes–regulatory pathways with corresponding phenotypes is more challenging. The fusion of the manus and pelvis at such an early stage in avian evolution may have been caused by modifications in genes and/or developmental paths. The origin of flight has significantly changed the bauplan of birds (42). Given the potential functional benefits, the complete fusion in the manus and pelvis in some early birds, but only rarely occurring, may reflect the refinement of flight capability, suggesting developmental plasticity (43). This is not unusual, because environmentally (here, terrestrial locomotory changes to flight) induced morphologies are common in animals and some of them actually spur the evolutionary success of certain groups (44, 45). Our study also shows that the manus fusion occurred earlier than the pelvis in avian evolution. We posit that this pattern reflects that the forelimbs underwent greater selection pressure and thus modified more rapidly during the course of flight evolution.

In summation, *Pterygornis* records the oldest occurrence of a fused carpometacarpus and pelvis in birds, and it is premature to pick which of the abovementioned hypotheses or their combination are most compatible with the evolutionary pattern without additional knowledge about the development of these bones and the molecular–developmental mechanisms of extant birds. Future studies are necessary to test these hypotheses and pinpoint the developmental pathways involving the bone fusion in early avian evolution.

Materials and Methods

Bone Histology Preparation. The bone cross-sections were prepared following the standard methods (46). Four samples were taken from the positions as close to the middiaphysis as possible from the left humerus and femur, right ulna and radius (Fig. 1A). Samples were embedded in one-component resin (EXAKT Technovit 7200) and hardened in a light polymerization device (EXAKT 520) for 12 h. Histological cross-sections were cut using an accurate circular saw (EXAKT 300CP). Sections were glued to frosted glass slides with adhesive (EXAKT Technovit 7230) and then ground down using the EXAKT 400CS grinding system until the desired optical contrast was obtained. The bone sections were checked by light microscopy under normal and polarized lights (Zeiss AX10). Images were captured using a digital camera (Zeiss AxioCam MRc5).

Phylogenetic Analysis and Ancestral State Reconstruction. The phylogenetic analysis was performed using the modified dataset of Mesozoic birds in Wang et al. (7). Description and complete results of the phylogenetic analysis are provided in the *SI Text* and *SI Appendix*. To trace the evolutionary changes of the manus and pelvis fusion across a broad phylogenetic scale, a paravian tree was constructed (Fig. 3 and Fig. S6). The phylogeny of Mesozoic birds is modified from the reduced strict consensus resolved in this study, and the placements of the major nonavian paravian clades follow Turner et al. (27). We formulated five binary characters to describe the degree of fusion of the pelvis and the alular and major metacarpals in paravians (*SI Text*). Since the pelvis and manus fusions rarely occur in nonavian paravians and they are likely nonhomologous to these features in Aves, we only selected a few species from among those nonavian paravians and their closest relatives that provide unambiguous information regarding the states of bone fusion in question. The ancestral states of these five characters are reconstructed for the major nodes across the Paravian phylogeny. This analysis was conducted using the parsimonious method in the Mesquite software package (47), and the complete results are given in Fig. 3 and Fig. S6.

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