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Medulla-free barb rami highlight the morphological diversity of early feathers

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ABSTRACT

Recent advances have deepened our understanding of the evolutionary and developmental origins of feather branching architectures. However, the internal tissue differentiation within these branches has received limited attention. This study examined eight fossilized feathers preserved in early Late Cretaceous Burmese amber, characterized by barb rami composed entirely of cortical tissue with no internal medulla. Based on barb rami morphology, the feathers were categorized into three distinct morphotypes. Comparative analysis with feather development in extant chickens suggested minimal tissue differentiation in these early feathers. Functional simulations further revealed that modern barb rami configurations provide greater aerodynamic stability than medulla-free early feathers under most conditions, highlighting flexural stiffness as a key factor in the evolution of feather branches. The presence of medulla-free barb rami suggests that although the three-level hierarchical branching pattern characteristic of modern feathers had emerged by the Jurassic, tissue differentiation within feather branches remained developmentally unstable during the Late Cretaceous. This instability likely contributed to the structural variability of early feathers, enabling morphologies that no longer persist in modern birds.

Keywords: Barb; Amber; Late Cretaceous; Evo-Devo; Barbules; Medulla; Myanmar

INTRODUCTION

Modern feathers represent one of the most architecturally elaborate integumentary innovations in vertebrate evolution, distinguished by hierarchical branches of rachis, barbs, and barbules (Lucas & Stettenheim, 1972; Prum & Dyck, 2003). In the typical bipinnate feather, the central rachis (feather shaft)

extends from the proximal calamus and divides the feather into two vanes (Prum & Dyck, 2003). The primary branches of the rachis, called barbs, each comprise a central ramus that supports paired rows of secondary branches, known as barbules (Figure 1A, B) (Lucas & Stettenheim, 1972; Prum, 1999; Prum & Dyck, 2003).

In modern feathers, the rachis and barbs exhibit a composite architecture typified by a dense peripheral cortex surrounding a spongy internal medulla (Chang et al., 2019; Lucas & Stettenheim, 1972). This sandwich-like cross-sectional structure (Figure 1C) provides flexibility and rigidity, contributing to the aerodynamic and mechanical functionality essential for flight, in addition to thermoregulation, and display (Chang et al., 2019; Wang et al., 2020). Despite extensive research on the evolutionary and developmental mechanisms underpinning feather branching patterns (Prum, 1999; Prum & Brush, 2002; Prum & Dyck, 2003; Xu, 2020; Xu & Barrett, 2025; Xu & Guo, 2009), the structural variations of feather branches have received relatively little attention (Chang et al., 2019; Wang et al., 2020).

Recent paleontological evidence has documented Cretaceous feathers exhibiting medulla-free rachises, attributing them to incomplete development of the cylindrical shaft (Wang et al., 2020). This underscores the importance of radial tissue differentiation within the follicle in driving early feather morphology (Wang et al., 2020). Although it remains unclear whether these structures reflect developmental anomalies or transient evolutionary variants, their prevalence across both non-avian and avian theropods, including in the summer plumage of extant penguins (Wang et al., 2020), suggests a potential functional role in promoting early feather diversity. Such structural differences may have supported ecological adaptation, raising important questions about the interplay between internal tissue differentiation and branching architecture in driving the morphological diversification of early feathers.

Given that the rachis forms through the fusion of barb ridges during morphogenesis (Prum, 1999), it has been speculated that medulla-free barb rami may have existed in early feathers, although direct fossil evidence for such structures

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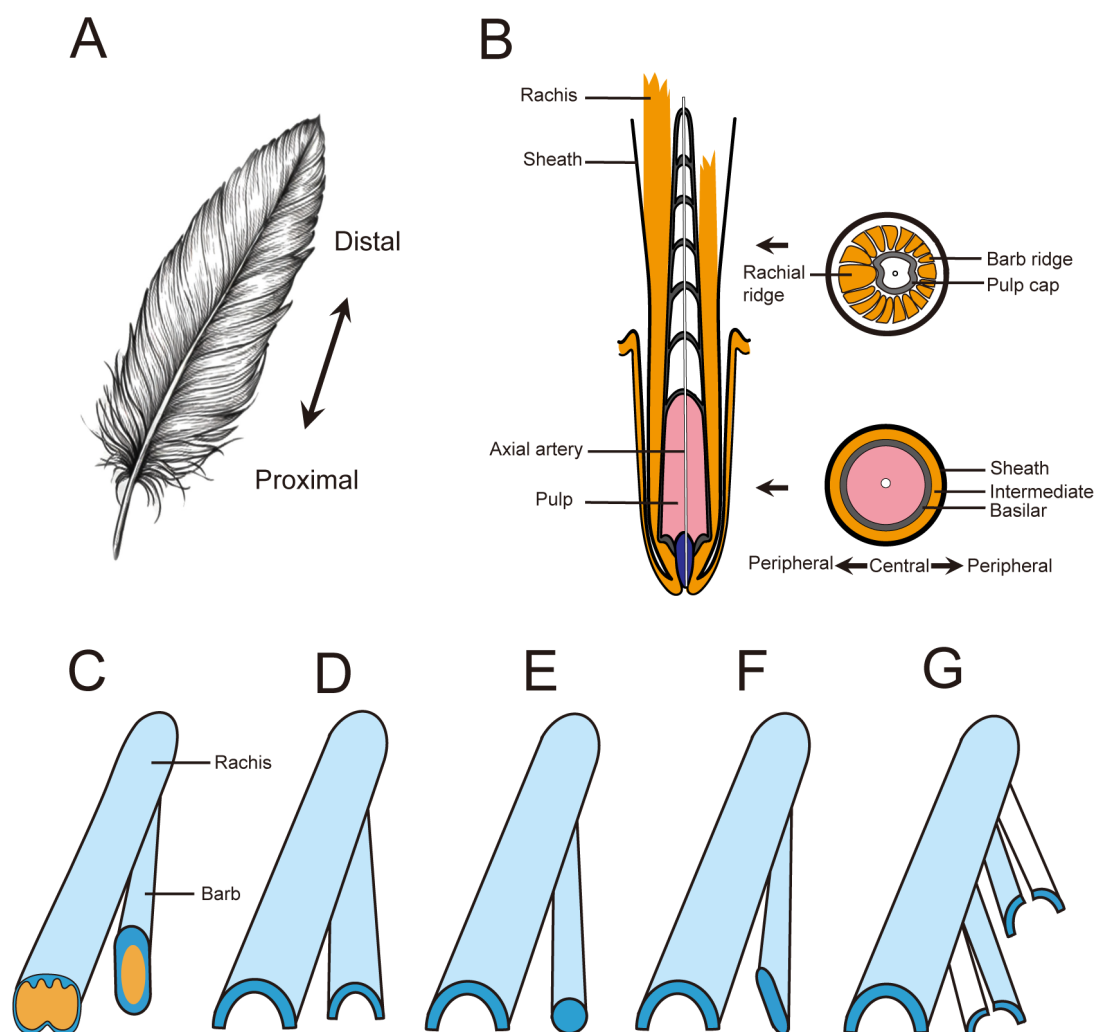


Figure 1 Schematic representation of feather structures

A: Diagram showing the overall morphology and orientation of an extant flight feather. B: Sagittal cross-section of a developing flight feather, cut through the rachial ridge (left), alongside horizontal cross-sections at different levels of the growing feather (right) (modified from Lucas & Stettenheim (1972)). C–G: Illustrations of distinct patterns of rachial and barb tissue differentiation, along with their modes of attachment to the rachis: C: Sandwich-like barbs connected to a similarly structured rachis, typical of modern feathers, featuring an internal spongy medulla (yellow) surrounded by a cortical layer (dark blue). D: Ribbon-like barbs attached to a ventrally open rachis that lacks both a medulla and ventral cortex, retaining only a dorsal cortex. E: Beam-like barbs associated with a ventrally open rachis, representing a primitive condition with limited dorsal cortex differentiation and absence of other specialized tissues. F: Plate-like barbs linked to a ventrally open rachis, composed entirely of an expanded cortex without a distinct medulla. G: Ventrally open rachis bearing split barbs that form a mesh-like structure. Not to scale.

has been lacking, even in feathers with ventrally open rachises (Wang et al., 2020). Here, we describe a series of morphologically enigmatic feathers preserved in early Late Cretaceous (approximately 99 million years ago (Ma)) Burmese amber (Shi et al., 2012), characterized by ribbon-like medulla-free barb rami attached to ventrally open rachises. Detailed examination suggested that some of these ribbon-like barbs were ventrally open, with the rami split along their midlines in one specimen, forming a unique mesh-like network between adjacent barbs (Figure 1G), an arrangement unknown in both extant and fossil birds.

This study aimed to (1) document the morphology of medulla-free barb rami in Burmese amber feathers, (2) investigate their structural, developmental, and functional implications, and (3) explore the potential role of incomplete tissue differentiation in the early evolution of feather morphology. These analyses contribute to a more comprehensive understanding of how tissue differentiation,

alongside branching patterns, shaped the evolutionary transition from early feathers to modern feather architectures.

MATERIALS AND METHODS

Feather sampling

All amber-embedded feather specimens analyzed in this study were kindly donated in 2016 by an anonymous private collector to the Biology Museum of East China Normal University (ECNU), Shanghai, China. None were associated with the Myanmar Economic Corporation, ensuring compliance with the moratorium issued by the Society of Vertebrate Paleontology (Haug et al., 2020). The specimens were collected from early Late Cretaceous deposits in Myanmar, dated to approximately 99 Ma (Shi et al., 2012).

For histological analysis, two regenerating feathers were sampled from a one-year-old White Leghorn chicken (*Gallus gallus*) maintained at the ECNU animal center. The first was a

fifth primary remex undergoing active regeneration, approximately four weeks post-plucking and representing one fourth of its full growth, with the developing portion embedded within the follicle collected for developmental analysis. The second sample was an early-stage developing covert contour feather from the breast region. To label proliferating cells, the chicken was intravenously injected with 1% bromodeoxyuridine (BrdU, Sigma B5002, USA) 3 h prior to feather extraction. Feather collection and paraffin sectioning were performed following protocols approved by East China Normal University (Approval Number ARXM2024010).

A fully developed breast contour feather with open vanes was extracted from the same chicken for functional analysis. This feather was selected due to its structural similarity to early fossil feathers, particularly its lack of barbule hooklets, making it an appropriate analog for aerodynamic analysis.

The flight feather was selected for developmental analysis due to its larger size and well-characterized molecular and morphological features (Chang et al., 2019), facilitating detailed visualization of barb ramus development. In contrast, the smaller size of contour feathers makes it more challenging to observe fine-scale developmental features. Nevertheless, previous studies have demonstrated that covert contour feathers share similar morphogenetic trajectories with flight feathers (Wang et al., 2020), despite differences in shape and aerodynamic function. For functional simulations, however, contour feathers were used instead of flight feathers, as the latter possess interlocking barbules that could skew aerodynamic results. Given the focus of this study on the barb morphology of contour-like primitive feathers, extant non-interlocking contour feathers serve as a more appropriate functional analog.

Terminology

Terminology describing feather structures and orientations followed Lucas & Stettenheim (1972), while descriptions of developing follicle adhered to Prum (1999). Specifically, “proximal” and “distal” refer to positions closer to or farther from the calamus (Figure 1A), respectively, while “central” and “peripheral” denote structures near or far from the follicle center in cross-section (Figure 1B), respectively. The terms “dorsal” and “ventral” are defined based on cross sections of a fully-open feather when the superior umbilicus faces ventrally; “dorsal” refers to the upper side of the feather structures in this orientation, and “ventral” indicates the lower side. Descriptive terms such as “sandwich-like”, “ribbon-like”, “beam-like”, and “plate-like” characterize different barb ramus configurations. “Sandwich-like” refers to the cross-sectional structure typical of modern feathers, where an internal spongy medulla is enclosed by a cortex layer (Figure 1C). In contrast, “ribbon-like”, “beam-like”, and “plate-like” describe overall barb ramus morphologies. “Ribbon-like” barbs have only a dorsal cortex, with some differentiation dorsally but lacking a distinct medulla and ventral cortex (Figure 1D, G). “Beam-like” barbs represent the most primitive condition, showing minimal dorsal cortex differentiation and no other tissue specialization (Figure 1E), while “plate-like” barbs consist solely of an expanded cortex, without a distinct medulla (Figure 1F).

Histological sectioning and staining

The regenerating region of the chicken primary remex was embedded in paraffin and sectioned at 5 mm intervals from the immature proximal end to establish a growth series. After fixation in 4% paraformaldehyde for 4 h, samples were

dehydrated through a graded ethanol series (30%–100%). Paraffin sections (7 µm thick) were cut perpendicular to the longitudinal axis of the feather.

Hematoxylin and eosin (H&E) staining was conducted following the protocols of Fischer et al. (2008), while BrdU assays were performed according to Lee et al. (2001), using an AEC Immunohistochemistry Color Development Kit (Sangon Biotech, E670031, China) in place of the original chromogenic detection method. Masson staining was conducted following Suvik (2012). Histological slides were imaged using a Nikon Digital Sight 10 Camera System connected to a Nikon Ni-E transmitted microscope.

Micro-computed tomography (CT)

High-resolution micro-CT was used to image amber-embedded fossil feathers. Specimen ECNU A32 was scanned at the Molecular Imaging Center, University of Southern California, using a Phoenix v-tome-x industrial CT scanner (beam energy: 18 kV, resolution: 4.0 µm per pixel). ECNU A143-1 was scanned using the same instrument at Shanghai Yinghua Inspection & Testing (beam energy: 12 kV, resolution: 3.9 µm per pixel). Segmentation was performed using Mimics v15.0 and Amira-Avizo v2020.2 at the University of Southern California.

Functional simulation

To evaluate the flexural stiffness and aerodynamic properties of feathers with different barb ramus morphologies, computational wind tunnel simulations were performed using ANSYS FLUENT v.12.0 (Ansys Inc., 2009). Physical wind tunnel testing was not employed due to the limitations of current three-dimensional (3D) printing technologies, which are unable to accurately reproduce fine feather structures such as individual barbs and barbules. While empirical validation remains valuable, previous studies have shown that simplified digital models can reliably capture key aerodynamic properties of feathers (Colognesi et al., 2021; Wang et al., 2020).

3D feather models were reconstructed from micro-CT data to compare the fossil specimen ECNU A32 with a modern contour feather featuring open vanes, extracted from a one-year-old White Leghorn chicken (Supplementary Figure S1A, B). For computational feasibility, barbules were omitted from all models. This simplification is supported by prior findings suggesting that although barbules contribute to drag, they exert minimal influence on the primary aerodynamic behavior of the vane (Colognesi et al., 2021).

To systematically examine how barb ramus morphology affects aerodynamic performance, a series of simplified models with distinct cross-sectional shapes were constructed (Supplementary Figure S2): Model I: A ribbon-like barb with a crescent-shaped cross-section, simulating ventrally open barb rami of morphotype A (Supplementary Figures S2A, S3A); Model II: A ribbon-like barb with paired half-crescent cross-sections, representing split rami formed by adjacent barbs in morphotype B (Supplementary Figures S2B, S3B); Model III: A plate-like barb with a 10:1 (length-to-width ratio) flat elliptical cross-section, reflecting morphotype C (Supplementary Figures S2C, S3C); Model IV: A hollow barb with a circular cross section, representing an idealized, non-biological form (Supplementary Figures S2D, S3D); Model V: A hollow barb with a 5:1 (length-to-width ratio) elliptical cross-section, reflecting the structure of modern flight feather rami (Supplementary Figures S2E, S3E). To assess the impact of

hollowness on aerodynamic performance, two additional models were included: a perfectly solid barb with round cross-section (Model VI, Supplementary Figures S2F, S3F) and a solid barb with a 5:1 (length-to-width ratio) elliptical cross-section (Model VII, Supplementary Figures S2G, S3G). Structural dimensions and modeling parameters for all models are provided in Supplementary Figure S3.

All models incorporated a fixed, cylindrical rachis for consistency, with only the barbs allowed to respond to aerodynamic forces. A complete feather vane was not simulated, and interlocking barbules were intentionally excluded from the analysis to isolate the aerodynamic contributions of the rami themselves.

Fluid-structure interaction (FSI) analysis

One-way FSI analysis was conducted to evaluate barb deformation under aerodynamic loading and to examine how these deformations influence aerodynamic performance (Bektaş et al., 2020; Lim & Xiao, 2019). The one-way FSI approach ignores the influence of structural deformation on airflow while effectively capturing the interaction between the flow and structure, ensuring high computational efficiency. This method is particularly well-suited for preliminary analyses of complex biological structures such as covert feathers (Wu et al., 2023). The simulation procedure included three stages: 1) modeling external airflow around the feather structure; 2) mapping the flow field results to the structural model; and 3) performing a one-way coupled simulation.

Material properties were assigned based on experimentally measured values for feather keratin. A density of 1.15 g/cm³ was used (Hertel, 1966), along with a Young's modulus of 2.5×10^9 N/M² (characterizing the material stiffness against the external force) (Bonser & Purslow, 1995; Macleod, 1980) and Poisson's ratio of 0.45 (Sullivan et al., 2017). Each model feather measured 60 mm in length, with barbs 10 mm long, extending from the rachis at a 45° angle (Supplementary Figure S2A). The rachis was modeled as a cylinder with a diameter of 2 mm. Barb rami had a diameter of 1 mm, with a cortex thickness of 0.05 mm for hollow models (Supplementary Figure S3). Ventrally open barb rami of morphotype A were modeled as longitudinal halves of a hollow cylinder (Supplementary Figure S3A), while the paired branches of morphotype B corresponded to quarter-cylinder segments (Supplementary Figure S3B).

To evaluate aerodynamic performance under different conditions, each morphotype was subjected to two airflow orientations: (1) perpendicular to the feather vane, representing lateral wind conditions when covert contour feathers are lifted by airflow; and (2) parallel to the rachis from the calamus, simulating aerodynamic behavior in headwind conditions when covert contour feathers remain tightly appressed to the body surface during flight. The second scenario closely reflects the natural aerodynamic function of covert contour feathers, which primarily streamline airflow along the body.

While the simulated forces may not directly replicate real-world aerodynamic loads, relative displacements from the static state provide meaningful indicators of flexibility and aerodynamic efficiency across feather morphologies. Due to the pronounced differences in elasticity between the cortex and medulla, the flexural stiffness of feather barbs is primarily determined by cortical geometry (Bachmann et al., 2012; Bonser & Purslow, 1995; Purslow & Vincent, 1978). These

simulations offer insight into how primitive feather structures influenced aerodynamic performance, informing broader interpretations of feather evolution and the origins of aerodynamic function.

RESULTS

Morphotypes

Eight feathers preserved in seven Burmese amber inclusions were included in this study (Figures 2–6; Supplementary Table S1). Although rachial morphology varied among and along individual feathers, all specimens exhibited a ventrally open rachis to some extent. For example, ECNU A143-1 (Figure 2A) retained an open rachis along its entire length, similar to CNU A0005 (Wang et al., 2020), while in ECNU A147, the open region was restricted to the proximal half (Figure 2J). As the focus of this study was on barb structure rather than rachial and barbule morphology, the feathers were categorized into three distinct morphotypes based on barb ramus conformation.

Morphotype A (Figures 2–3; Supplementary Video S1) is represented by four amber specimens: ECNU A49, A143, A147, and A148. ECNU A147 and A148 each contained a nearly complete feather with symmetrically distributed barbs and barbules (Figure 2E–N). ECNU A143 included two isolated feathers, designated as ECNU A143-1 (Figure 2A–D; Supplementary Video S1) and A143-2 (Figure 3A–D), respectively. ECNU A49 consisted of a single barb (Figure 3E–G), limiting morphological interpretation. All morphotype A feathers were characterized by proximodistally broad, ribbon-like barb rami bearing barbules along both proximal and distal edges. CT reconstructions revealed that these rami consisted solely of a thin dorsal cortex, lacking both medulla and ventral cortex (Supplementary Figure S4). This configuration produced ventrally open rami with a crescent-shaped cross-section not observed in modern birds. Unlike previously described ventrally open rachises, which typically include a midline ridge and lateral flanges (Wang et al., 2020), the ribbon-like rami in morphotype A lack such reinforcing structures, suggesting enhanced flexibility (see “Structural stability of different barb rami”).

Ribbon-like morphology occurred either within specific regions of individual barbs or across multiple barbs along a single shaft. In ECNU A148 (Figure 2N; blue arrowheads), ribbon-like and solid beam-like segments alternated along the same barb, whereas in A143-1 (Figure 2A, O), most barb rami were proximally ribbon-like and transitioned into solid beams distally. These patterns likely reflect variation in dorsal cortex expansion during barb development. As such, morphotype A feathers are defined by the presence of ventrally open, ribbon-like barb rami bearing barbules along proximal and distal edges.

Morphotype B (Figures 4–5; Supplementary Video S2) is represented by a single isolated feather (ECNU A32), characterized by symmetrically arranged barbs and a relatively thick rachis (Figure 4A–I). CT scans revealed a ventral cleft in the rachis, producing a V-shaped cross-section (Figure 5C). Barbs located in the proximal and distal regions were beam-like, while those in the central portion of the rachis bifurcated shortly after emerging (Figure 5A, E). For the bifurcated barbs, each branch corresponded to one half of the crescent-shaped dorsal cortex of morphotype A barb rami (Figure 5D, F; Supplementary Video S2). The two branches of

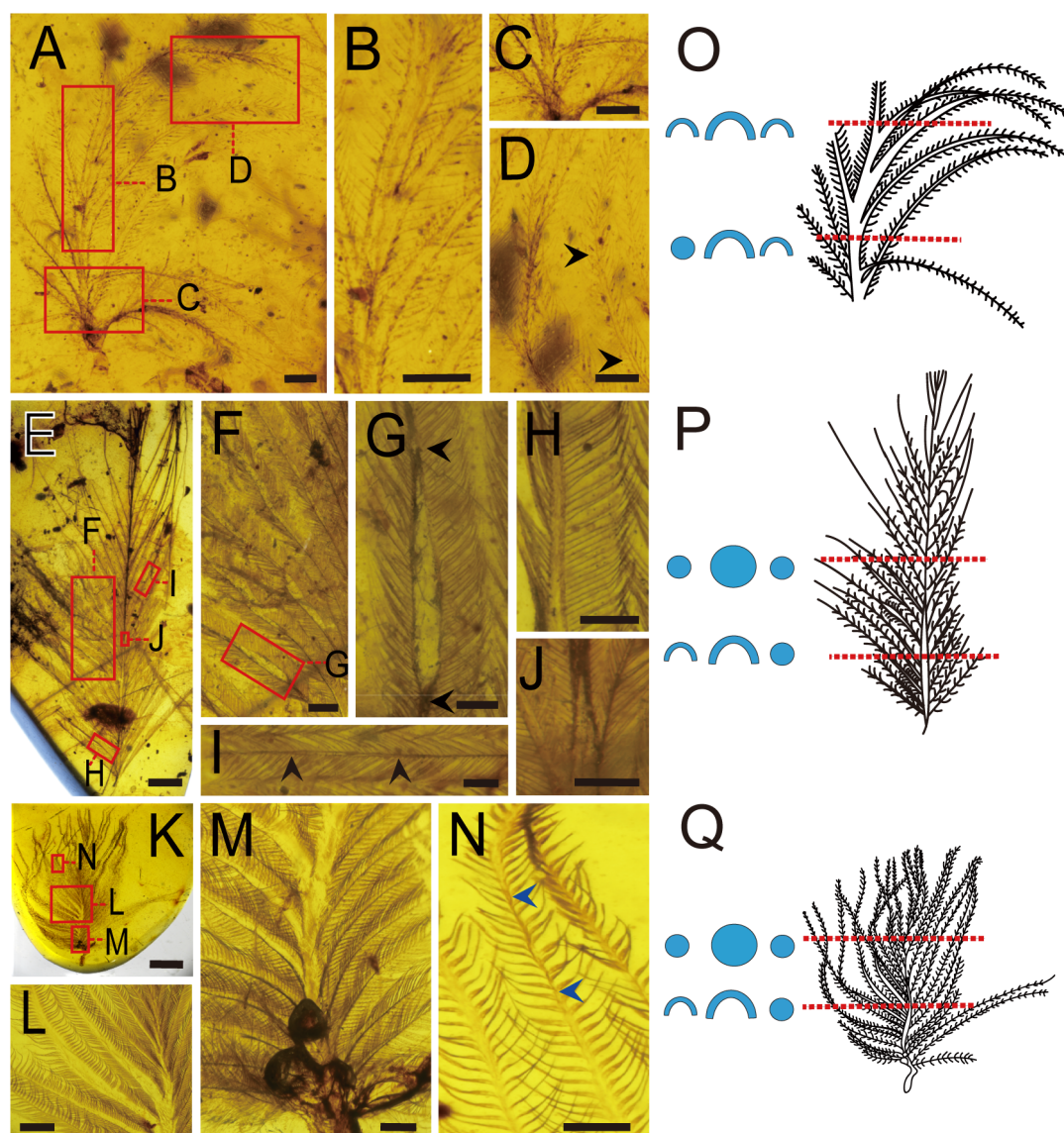


Figure 2 Morphotype A feathers showing ventrally open barb rami

A–N: Photographs of isolated feathers displaying ventrally open barb rami: A–D: ECNU A143-1; red boxes in A indicate regions magnified in B–D. E–J: ECNU A147; red boxes in E and F indicate areas shown in G–J and H, respectively. K–N: ECNU A148; red boxes in K mark regions shown in L–N. Paired black arrowheads in D, G, and I indicate the location of ventrally open barb rami; paired blue arrowheads in N denote solid, beam-like barb rami. O–Q: Schematics illustrating representative feather morphologies (right) and corresponding cross-sections of rachis and barb rami (left) at selected positions. Red dashed lines indicate cross-section planes, blue silhouettes represent solid (circle) or ventrally open (crescent-shaped) rachial and barb cross-sections on either side of the rachis (not to scale). Scale bar: 0.5 mm for A, E, F, and M; 0.2 mm for B–D, G–J, and N–L; and 1.5 mm for K. O–Q are not to scale. Photos were taken by Y.Y. Zhang.

a single barb fuse distally to form a solid beam, whereas proximally, a single branch from each adjacent barb fuses to form a plate where they incorporate into the rachis, creating a unique mesh-like structure between barbs (Figure 5E, F). Notably, barbules are absent along the outer margins of the branches immediately distal to the plate, where the barbules are oriented toward the center (Figure 5A–F). This arrangement demonstrates that the plate is formed by the fusion of split halves from adjacent barb rami (Figure 5E, F), a feature not seen in fossil or modern feathers. In cross-section, the distal fork branch was consistently positioned ventrally to the proximal branch as they merged into the rachis (Figure 5C), giving the appearance of a helical twist immediately after branching from the feather shaft. Thus, morphotype B feathers can be distinguished by the presence of mesh-like network between adjacent barbs.

Morphotype C (Figure 6) is represented by two specimens, ECNU A101 (Figure 6A–E) and A149 (Figure 6F–J), each containing a single feather with symmetrically distributed barbs and barbules. In contrast to morphotypes A and B, the barb rami in morphotype C feathers were proximodistally compressed and dorsoventrally expanded (Figure 6D, I). These rami lacked a medulla, and barbules were present along both the distal and proximal edges. These features distinguish morphotype C from other morphotypes.

Structural stability of barb ramus morphologies

Computational analyses demonstrated that barb rami with perfectly circular hollow cross-sections possessed the greatest structural stability, whereas those with elliptical cross-sections exhibited reduced flexural stiffness (Figures 7–9; Supplementary Tables S2–S4). These findings reinforce the

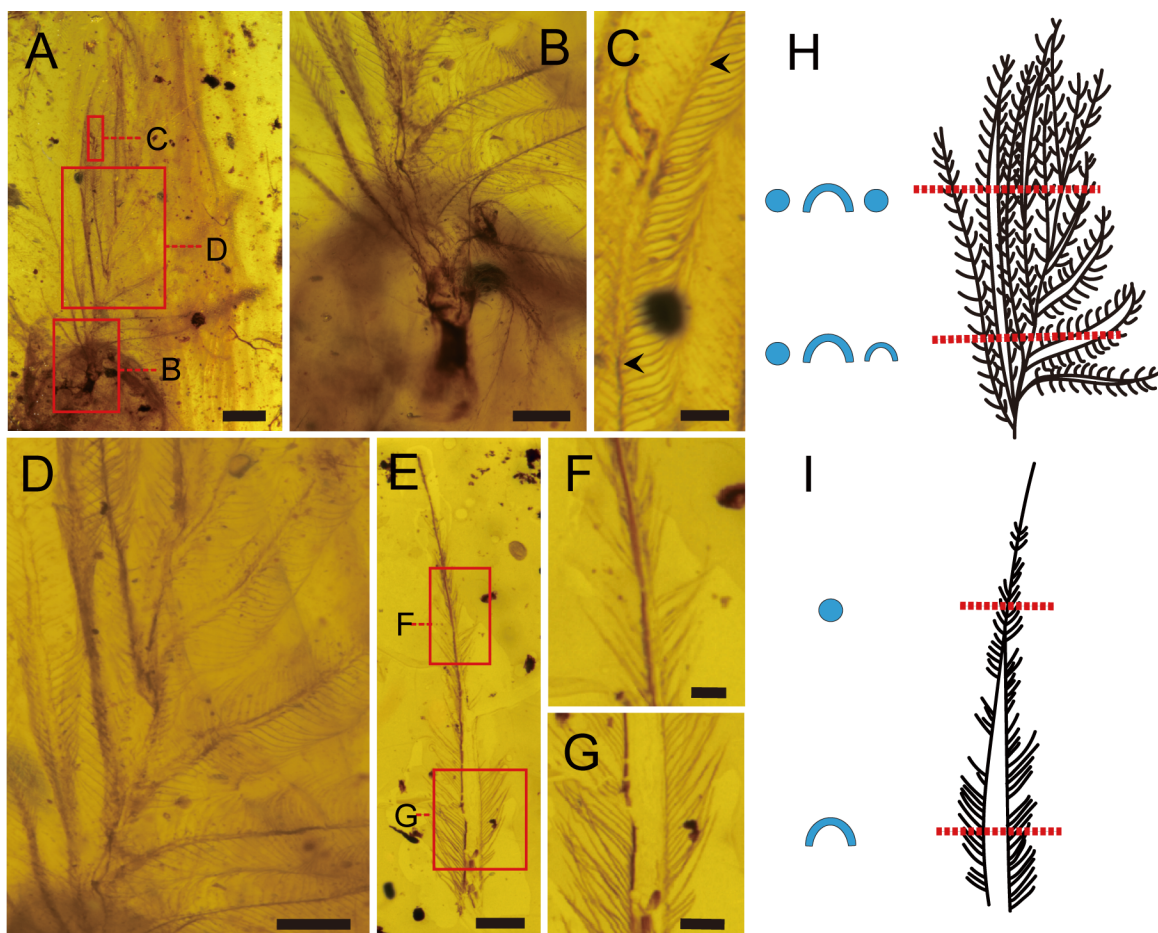


Figure 3 Morphotype A feathers showing ventrally open barb rami

A–G: Photographs of isolated feathers showing ventrally open barb rami: A–D: ECNU A143-2; red boxes in A indicate regions shown in higher magnification in B–D. E–G: ECNU A49; red boxes in E indicate areas shown in F and G. Paired black arrowheads in C mark the location of ventrally open barb rami. H, I: Schematics illustrating overall feather morphologies (right) and cross-sections of rachis and barb rami (left) at various positions along the corresponding feather (H) or barb (I). Red dashed lines indicate cross-section planes, blue silhouettes represent ventrally open (crescent-shaped) rachial and barb cross-sections (not to scale). Scale bar: 0.5 mm for A and E; 0.2 mm for B, D, and G, 0.1 mm for C and F. H, I are not to scale. Photos were taken by Y.Y. Zhang.

conclusion that flexural stiffness primarily depends on cross-sectional geometry rather than the intrinsic material properties of feather keratin (Bonser & Purslow, 1995; Chang et al., 2019; Wang & Meyers, 2017).

Specifically, barbs with round hollow cross-sections demonstrated maximal resistance to deformation when airflow moved along the vane axis from the calamus (Figure 8D). In contrast, under perpendicular airflow, forked barbs with paired half-crescent cross-sections exhibited enhanced strength (Figures 7B, 9A), attributable not to individual ramus geometry but to the formation of a mesh-like structure that reinforced stability under perpendicular airflow. When subjected to lateral airflow, barb rami with a 10:1 (length-to-width ratio) flat elliptical cross-section exhibited less displacement than those with a 5:1 (length-to-width ratio) elliptical cross-section (Figure 7H), likely due to a reduced windward surface area. However, under airflow from the calamus, the 10:1 elliptical barbs experienced greater displacement (Figure 8H), likely due to their larger windward area.

Overall, barb rami with a 5:1 elliptical cross-section, resembling modern feather barbs, emerged as among the least structurally stable morphologies (Figures 7H, 8H). Despite this limitation, modern feathers with closed vanes compensate for this structural limitation by interlocking into

vanes via hooklets, forming a mechanically superior structure compared to the mesh-like connections in ECNU A32. This interlocking arrangement enhances strength while minimizing weight.

Formation of typical feather barb

A barb, the primary branch of a feather, consists of a ramus lined with rows of barbules on either side (Prum & Dyck, 2003). The cross-sectional shape of barbs varies across different feathers and even along a single feather, such as covert contour feathers (Mueller & Gibson, 2023). While most extant feather barbs exhibit a proximodistally compressed, sandwich-like cross-section with an outer cortex and an internal medulla, the presence and extent of medullary development differ by species and feather type.

Barb development follows a highly coordinated morphogenetic sequence involving elongation of the filament and cellular differentiation of barb structures (Figure 10A) (Lin et al., 2020; Prum & Dyck, 2003). Development begins with the formation of a few barb ridges on either side of the rachial ridge, followed by the sequential emergence of additional ridges extending toward the new barb locus, which lies opposite, though not strictly aligned with, the rachial ridge (Figure 10B; Stage I). Unlike the stationary rachial ridge, barb

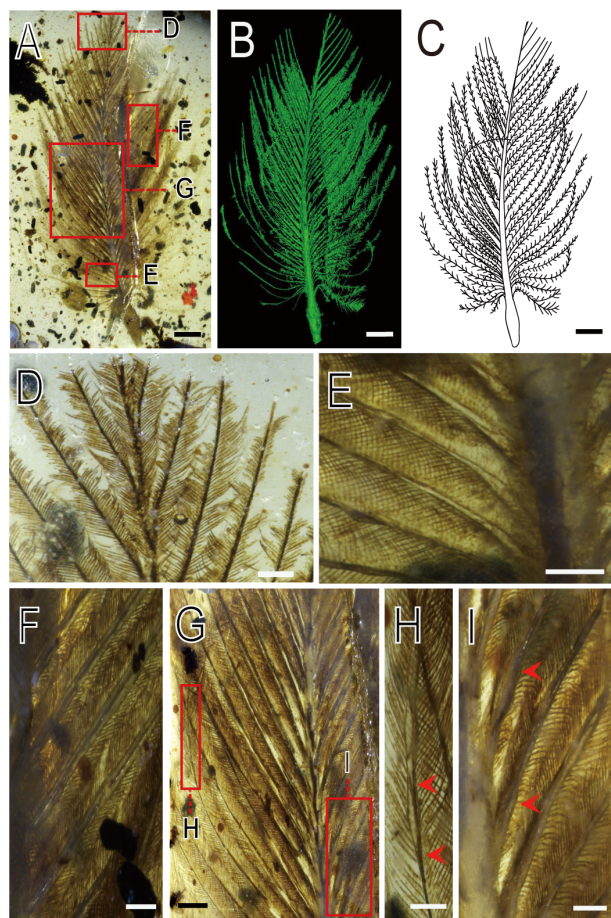


Figure 4 Morphotype B feathers showing split, ventrally open barb rami

A: Photograph; B: 3D segmentation; C: Schematic of isolated feather ECNU A32. Red boxes in A indicate regions shown at higher magnification in D–G, while red boxes in G indicate areas shown at higher magnification in H and I. Paired red arrowheads in H and I mark positions where the barb rami are ventrally open. Scale bar: 1.25 mm for A–C and 0.2 mm for D–I. Photos were taken by Y.Y. Zhang.

ridges undergo both axial (parallel to the longitudinal axis of the follicle) and tangential movement (perpendicular to the longitudinal axis of the follicle) during development (Lucas & Stettenheim, 1972; Prum, 1999).

Axially, in cross-sections taken perpendicular to the longitudinal axis of the follicle, all barb ridges appear at approximately the same developmental stage because they elongate at a uniform rate (Figure 10B). This synchrony enables spatiotemporal changes in feather formation to be tracked (Lucas & Stettenheim, 1972). Tangential movement influences ramus and barbule morphology along a single barb due to morphogen concentration gradients across the pulp periphery (Chang et al., 2019) (Figure 10B; Stages II, III). The combined axial and tangential movements produce a helical shift of barb ridges toward the rachial ridge (Lucas & Stettenheim, 1972), culminating in the integration of their proximal ends into the rachis (Figure 10B; Stage IV).

Histological assays, including H&E, Masson, and BrdU staining of serial cross-sections from developing chicken primary remex follicles, revealed that basilar cells located centrally within the epidermal collar were initially surrounded by a thick layer of intermediate cells (Supplementary Figure S5A–C). These cells subsequently reorganized into barb

ridges arranged radially along the follicle, separated by shallow furrows intercepted by pulp bulges (Supplementary Figure S5D–I). Shortly after ridge formation, differentiation into distinct barb components commenced. Within each barb ridge, a single basilar cell layer flanked the intermediate cells to form the marginal plates (Supplementary Figure S5D–I), while the remaining intermediate cells aggregated into an axial plate centrally positioned between barbule plates on either side, terminating at the ramus (Supplementary Figure S5F, I).

BrdU staining showed diffuse cell proliferation within the barb ridges, with BrdU-positive cells scattered throughout the epidermal basal layer, including barb ridge tips and furrows (Figure 10E–F; Stage I). As the ridges migrated tangentially, the barbule plates differentiated rapidly and underwent keratinization (Figure 10; Stage I). Apoptosis in the axial plate released mature barbules, whereas apoptosis in the marginal plates facilitated separation of adjacent barb ridges. Keratinization subsequently extended from the barbule plates to the peripheral walls, corresponding to the dorsal cortex of mature barb rami (Figure 10; Stage II). At this stage, BrdU staining revealed a high-proliferation zone along the basal epidermis (Figure 10E–F; Stage II), indicating coordinated inward growth of the barb ridges toward the follicle center, similar to the rachial ridge (Wang et al., 2020).

With continued development, expanding medullae displaced the barb rami toward the peripheral sheath, and keratinization progressed from the lateral cortex to the central walls, forming the ventral cortex of mature barb rami (Figure 10; Stage III). Cell proliferation declined, and the medullae completed keratinization as the peripheral walls of the rami attached to the sheath (Figure 10; Stage IV). The final cells to undergo keratinization were the basilar cells adjacent to the basement membrane, giving rise to the ventral cortex (Figure 10; Stage IV). A renewed zone of proliferative activity was detected along the thickened basement membrane, demarcating mature ridges from the pulp (Figure 10E–F; Stage IV).

While barb ridge development followed a spatiotemporal pattern similar to that of the rachial ridge (Chang et al., 2019; Wang et al., 2020), progressing radially from the periphery toward the center, histological comparisons revealed two key differences:

1. Keratinization in barb ridges began in the barbule plates, whereas in the rachial ridge, it initiated within the dorsal cortex;
2. The dorsal cortex of the rachial ridge remained in constant contact with the feather sheath throughout development, whereas in barb ridges, the peripheral walls (equivalent to the dorsal cortex of mature barb rami) were initially positioned central relative to the barbule plates and gradually shifted outward with medullary expansion.

DISCUSSION

Taphonomic effects

While ventrally open, medulla-free rachises have been reported in both carbonized compression fossils and Burmese amber specimens (O'Connor et al., 2012; Wang et al., 2020), the absence of medulla-free barb rami in any known avialan or non-avialan theropod specimens raises questions regarding whether these unusual barb structures represent genuine morphologies or are simply preservational artifacts. However, the structural composition of feather tissues offers important insights. Like the rachis, barb rami and barbules are

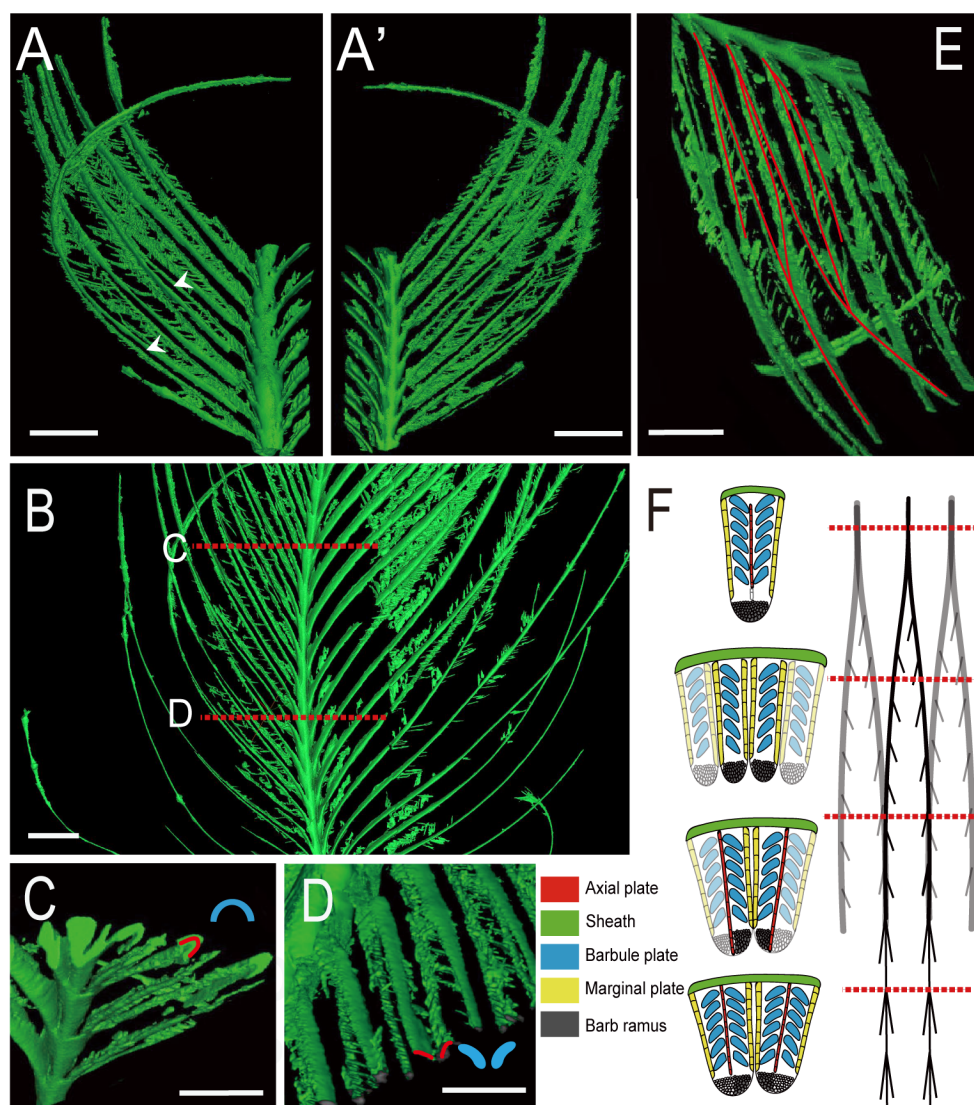


Figure 5 3D segmentation of ECNU A32 showing split barb rami

A, A': Ventral (A) and dorsal (A') views of 3D segmentation of ECNU A32; paired white arrowheads mark midline splitting of barb rami. B–D: Close-up views from various regions and angles illustrating split barb morphologies. Red dashed lines in B indicate locations of cross-sections shown in C and D; red outlines in C and D delineate cross-sectional profiles of medulla-free and split barb rami. E: Topological view highlighting bifurcated structure of split barb rami, with red outlines tracing individual branches. F: Schematic showing barb ridge configurations at different levels along split barb rami, illustrating morphological variation driven by temporal developmental shifts. Red dashed lines indicate cross-section planes. Scale bar: 1.25 mm for A–E. Images were taken by Y.Y. Zhang.

composed of keratin, a robust biomaterial highly resistant to aqueous degradation, organic solvents, and mechanical stress, even after death (McCoy et al., 2019; Moyer et al., 2016). This durability is evident in the well-preserved barb and barbule structures observed in amber-embedded feathers (Chang et al., 2019; Xing et al., 2018, 2020). Given this preservation resilience, it is unlikely that barbules, which are structurally more delicate than barb rami, would exhibit greater resistance to degradation than the medulla or ventral cortex. The consistent absence of these internal structures in fossilized barbs, similar to the loss of rachial medullae in some early feathers (Wang et al., 2020), suggests a developmental rather than taphonomic origin. Specifically, a heterochronic truncation of developmental processes may have inhibited the full formation of the medulla and ventral cortex, rather than these structures being lost through fossilization. In the case of morphotype B barbs, although the bifurcated rami could potentially result from taphonomic distortion, the distal fusion

of ramus branches is unlikely to be an artifact of preservation. These observations suggest that modifications in feather development, rather than taphonomic processes, more plausibly account for the structural configurations observed in fossilized specimens.

Structural stability of ventrally open feather barbs

Feathers bearing ventrally open barbs have not been documented in either fossil theropods or modern birds, distinguishing them from medulla-free rachises known from both extinct taxa and the summer plumage of extant penguins (Wang et al., 2020). This absence prevents direct investigations into their developmental regulation, such as transcriptomic differences compared to the sandwich-like barb rami of modern contour feathers, or their functional significance. The concurrent absence of hooklets in these early feathers, as observed in other Burmite amber specimens (Chang et al., 2019; Wang et al., 2020), combined with

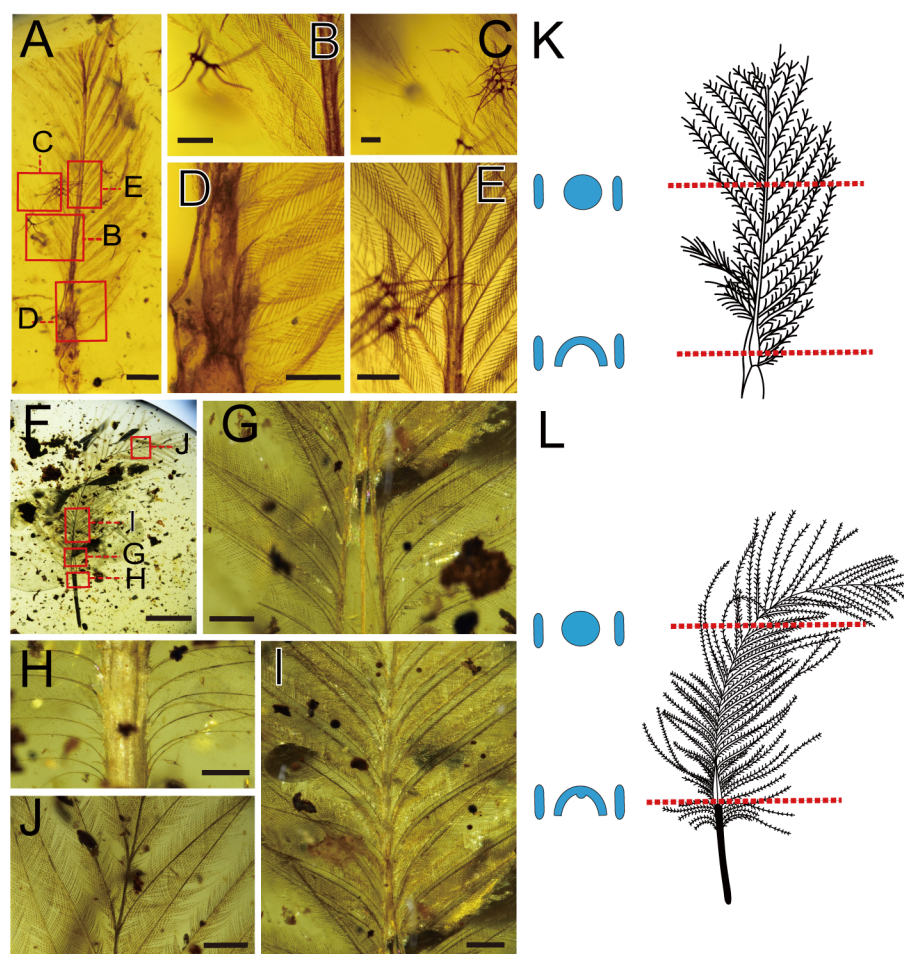


Figure 6 Morphotype C feathers showing medulla-free, plate-like barb rami

A–E: Photographs of ECNU A101 showing medulla-free, plate-like barb rami; red boxes in A indicate areas magnified in B–E. F–J: Photographs of ECNU A149; red boxes in F mark regions shown in G–J. K, L: Schematics depicting overall feather morphology (right) and cross-sections of rachis and barb rami (left) at different positions along the feather. Red dashed lines indicate cross-section planes, blue silhouettes represent solid (circular) or ventrally open (crescent-shaped) rachial cross-sections, as well as plate-like barb ramus cross-sections on either side of the rachis. Scale bars: 0.5 mm for A and G–I; 5 mm for F; 0.2 mm for B–E. K, L not to scale. Photos were taken by Y.Y. Zhang.

medulla-free and ventrally open barbs, implies limited aerodynamic stability. Given their small size and structural weakness, these feathers may have served a contouring function beneath larger covert or flight feathers, making their aerodynamic instability less consequential.

Instability of medulla-free, ventrally open barbs under parallel airflow (Figure 8H) suggests a propensity for vibration at certain flow velocities, further supporting their unsuitability for aerodynamic streamlining. This finding is consistent with the interpretation that animals bearing such feathers were unlikely capable of powered flight or gliding. In modern birds, closed-vaned regions of covert contour feathers possess hooklets, whereas open vanes lack both hooklets and ventrally open barb rami (Lucas & Stettenheim, 1972). Moreover, ventrally open barbs have not been observed in the feathers of flightless birds such as ostriches and emus (Gill et al., 2019), reinforcing the idea that this structural feature was eliminated during avian evolution. Collectively, these observations support the notion that not all phenotypic traits confer functional advantages or result from adaptive evolution.

However, functional simulations presented here highlight potential biomimetic applications. The bifurcated barb rami enhance structural stability by forming a mesh-like configuration, suggesting a potential design framework for

lightweight, mechanically resilient materials.

Putative developmental mechanisms underlying atypical barb rami

Although early feather development, including cellular dynamics within early barb ridges, appears broadly conserved relative to modern feathers (Chang et al., 2019; Wang et al., 2020), reproducing the distinct morphologies of early barbs within contemporary feather follicles remains technically unfeasible. Regulatory gene perturbations can globally alter feather structures (Ng & Li, 2018), yet fine-scale recapitulation of ancient barb ramus structures is constrained by intra- and inter-feather morphological variability. The lack of precise methodologies for spatially and temporally controlled gene expression in developing feather follicles further complicates experimental replication. In addition, the gene regulatory networks responsible for these unique morphologies may have decayed or disappeared entirely over evolutionary time (Chang et al., 2019). Consequently, the cellular and molecular events leading to the development of various ribbon-like barb rami in early feathers can only be inferred indirectly, based on the conserved processes observed in extant feathers, a common approach used in Paleo-Evo-Devo studies (Thewissen et al., 2012).

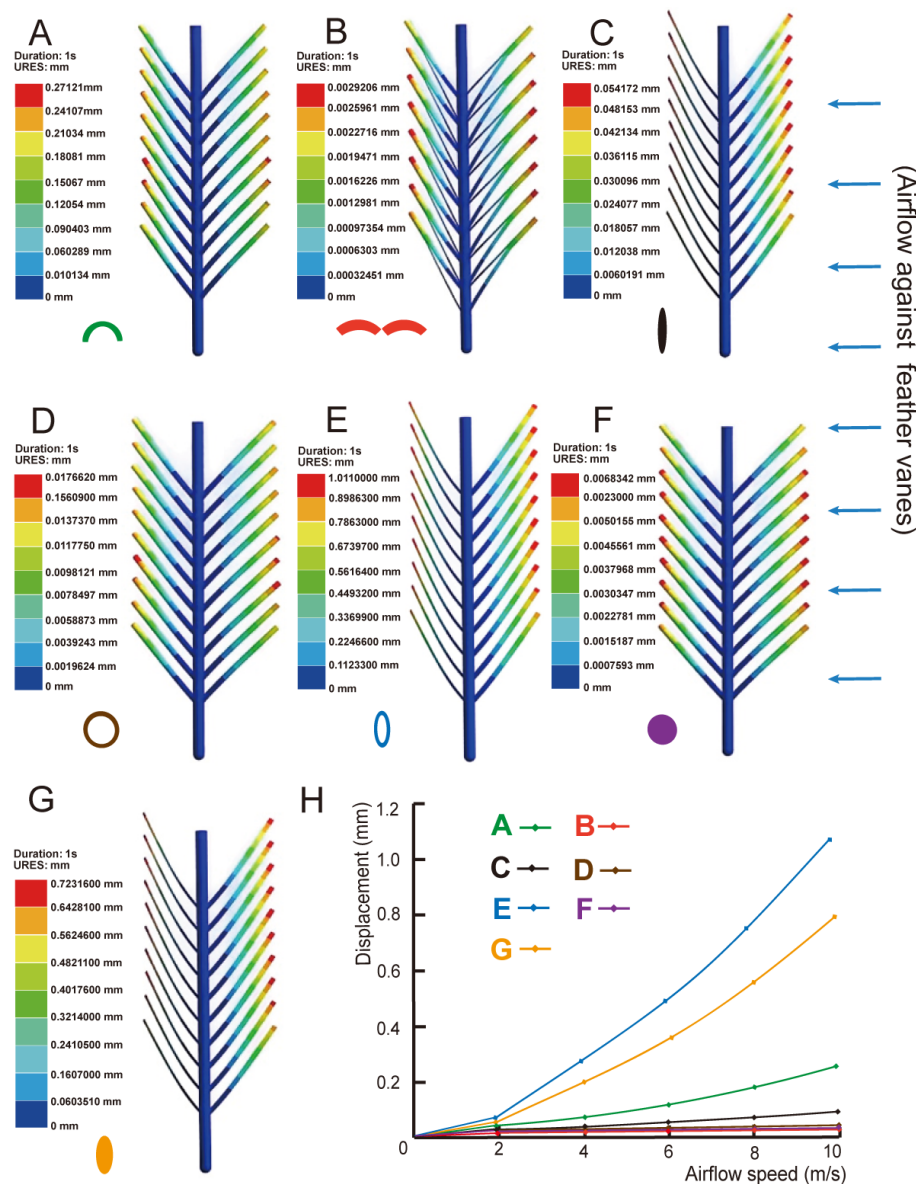


Figure 7 Simulated maximum displacement of feather barbs with varying cross-sectional shapes under perpendicular airflow to the feather vanes

Models simulate barb rami with the following cross-sectional shapes: A: Morphotype A with crescent-shaped cross-sections; B: Morphotype B with paired half-crescent cross-sections; C: Morphotype C with 10:1 flat elliptical solid cross-sections; D: Idealized, non-realistic tubular barb rami with perfectly round, hollow cross-sections; E: Modern flight feather with 5:1 elliptical hollow barb ramus cross-sections; F: Idealized, non-realistic tubular barb rami with perfectly round, solid cross-sections; (G) Idealized, non-realistic barb rami with 5:1 elliptical, solid cross-sections. Resultant displacement (URES) is expressed in mm, with symbols beneath each model representing corresponding cross-sectional shapes of barb rami. Comparative aerodynamic performance of simulated models is shown in H, with airflow speed on the x-axis and displacement on the y-axis. Results show that, under airflow perpendicular to feather vanes, barbs with paired half-crescent cross-sections (B, H) displayed the highest structural resistance, whereas those with 5:1 flat elliptical solid cross-sections (E, H) exhibited the greatest deformation. These findings underscore the critical role of cross-sectional geometry in determining barb flexibility and mechanical stability. Images were taken by J.W. Tang.

In morphotype A feathers, barb rami exhibit both ribbon-like and beam-like configurations depending on their position. The absence of the medulla and ventral cortex in ventrally open regions may reflect early truncation of proliferative activity, failed keratinization of medullary cells, or both, similar to patterns observed in medulla-free and ventrally open rachises (Wang et al., 2020). Under these conditions, mechanical reinforcement is limited to expansion of the dorsal cortex or possible increases in total barb diameter (Figures 7, 8), resulting in the ribbon-like barb rami. If a well-keratinized medulla could be formed, the solid beam-like barb rami would

not be necessary. In this context, solid beam-like barb rami are not indicative of more advanced differentiation but rather represent unexpanded dorsal cortex tissue (Figure 11A, Bii). Therefore, the presence of beam-like structures should not be interpreted as evidence of fully differentiated medullary or ventral cortex elements. The morphogenetic sequence in morphotype A feathers likely involves a temporal shift in tissue differentiation: (i) Initially, differentiation of the dorsal cortex alone results in a solid beam structure lacking expansion or additional tissue components (Figure 11Bii; tp1); and (ii) Subsequently, the dorsal cortex undergoes lateral expansion

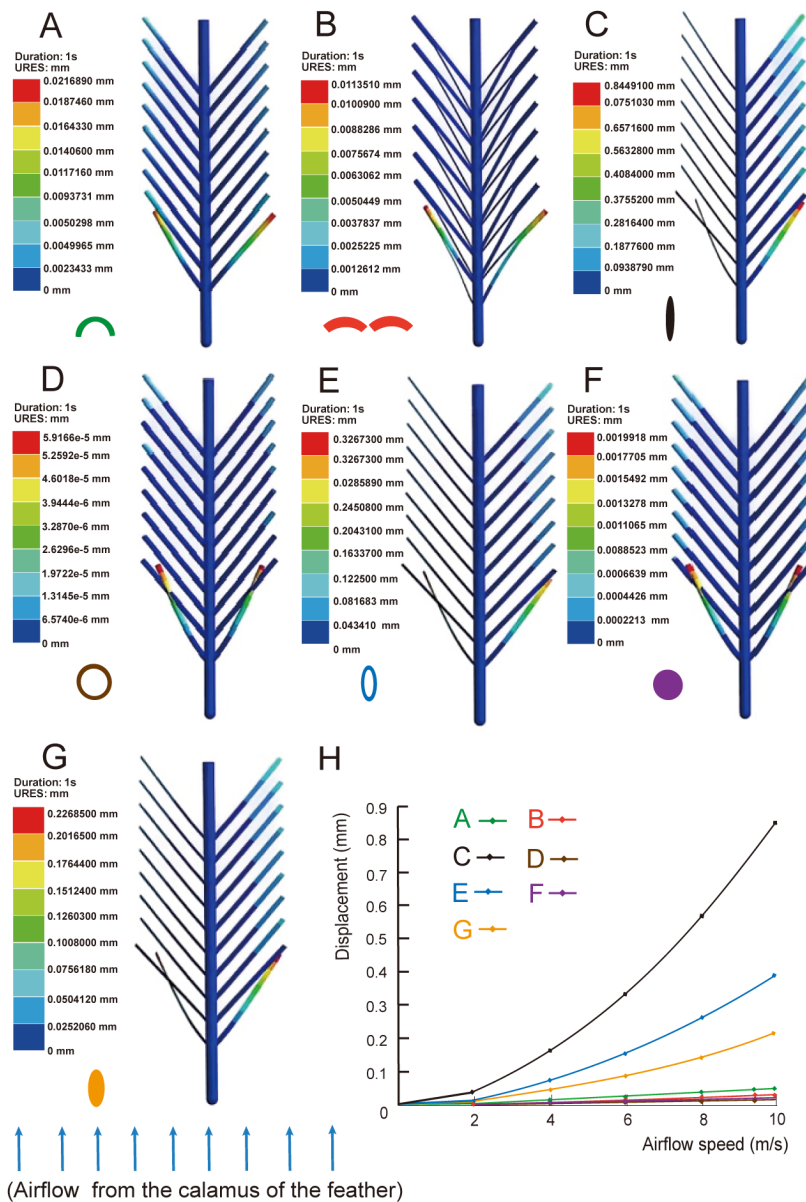


Figure 8 Simulated maximum displacement of feather barbs with varying cross-sectional shapes under airflow passing along feather vanes from the calamus

Models simulate barb rami with the following cross-sectional shapes: A: Morphotype A with crescent-shaped cross-sections; B: Morphotype B with paired half-crescents cross-sections; C: Morphotype C with 10:1 flat elliptical, solid cross-sections; D: Idealized, non-realistic tubular barb rami with perfectly round, hollow cross-sections; E: Modern flight feather with 5:1 elliptical hollow cross-sections; F: Idealized, non-realistic tubular barb rami with perfectly round, solid cross-sections; and (G) Idealized, non-realistic barb rami with 5:1 elliptical, solid cross-sections. Resultant displacement (URES) is expressed in mm, with symbols beneath each model representing corresponding cross-sectional shapes of barb rami. Comparative aerodynamic performance of simulated models is shown in H, with airflow speed on the x-axis and displacement on the y-axis. Results indicate that barbs with hollow circular cross-sections (D) exhibit the highest structural resistance, while barbs with solid elliptical 10:1 cross-sections (C) show the greatest deformation. These findings underscore the influence of cross-sectional shape on feather barb flexibility and stability under parallel airflow. Images were taken by J.W. Tang.

without concurrent formation or keratinization of the medulla, yielding ventrally open barb rami (Figure 11Bii; tp2). This pattern may correspond to stage II of developing sandwich-like barb rami in extant feathers (Figure 11A).

Unlike morphotype A, the morphogenesis of morphotype B feather ECNU A32 appears to have been more complex. Similar to other medulla-free feathers, the barb rami in this specimen lacked both medulla and ventral cortex. However, the presence of a bifurcated dorsal cortex implied that the axial plate, which separates the proximal and distal barbule

plates and typically terminates upon reaching the developing barb ramus, extended unusually far, reaching the apical tips of the developing barb ridges soon after the barb ramus morphogenesis initiated (Figure 5F, 11Biii). Apoptosis of these centrally extended axial plates not only produced split barb rami but also induced separation of the apical basilar cell population within each developing barb ridge into two discrete groups (Figure 5F, 11Biii). Later, the marginal plates, typically spanning the inter-ridge regions and extending to the apices of developing barb ridges, failed to reach these apical basilar

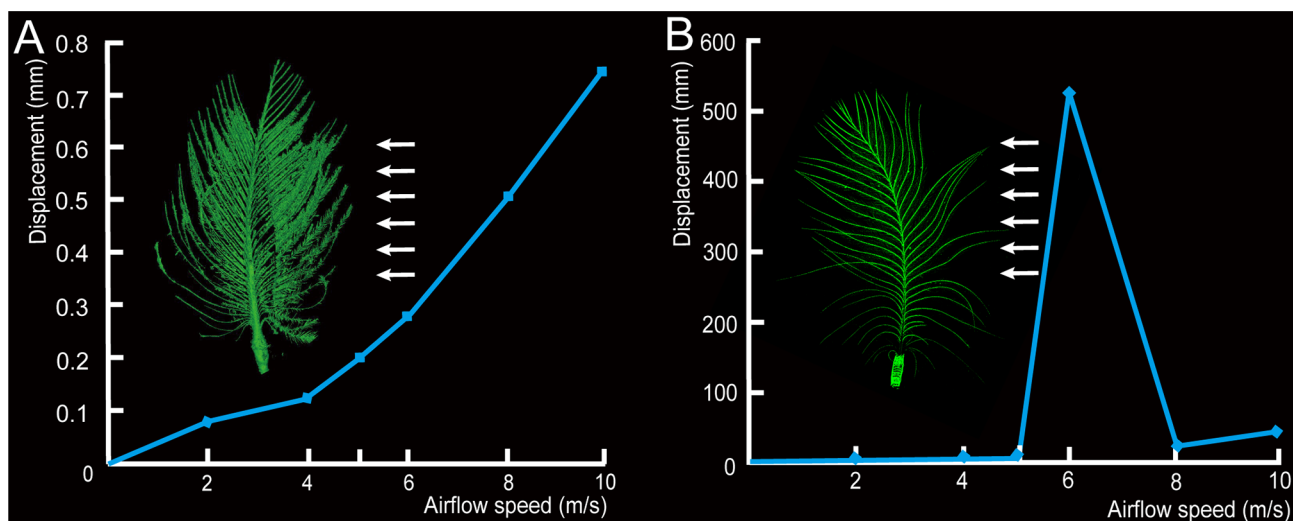


Figure 9 Aerodynamic performance of ECNU A32 (A) and an extant contour feather of comparable size and structure (B) under increasing airflow velocity (white arrows) directed against feather vanes

The x-axis represents airflow speed and the y-axis represents displacement. Extant contour feather experienced complete structural failure at 6 m/s, with displacement exceeding 550 mm. In contrast, ECNU A32 remained structurally stable across all tested airflow velocities, up to 10 m/s. These results suggest that the amber-embedded feather, characterized by forked barbs, exhibits greater mechanical strength compared to the modern contour feather. Images were taken by J.W. Tang and Y.Y. Zhang.

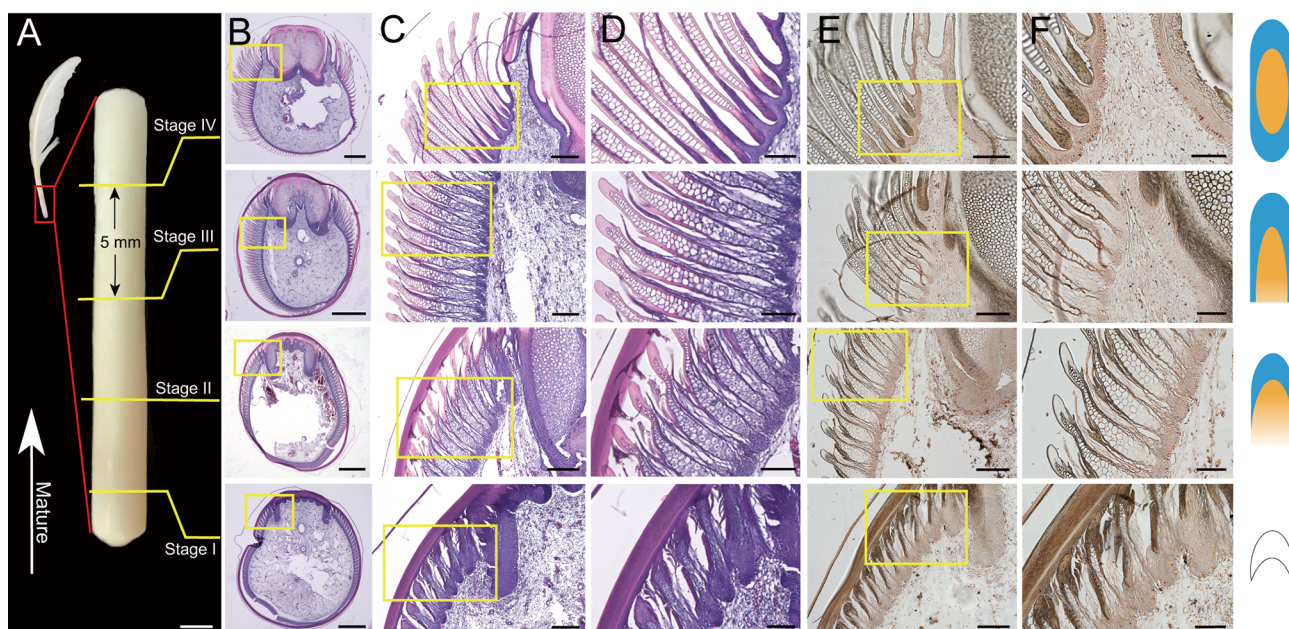


Figure 10 Tissue differentiation (H&E) and cell proliferation (BrdU) in a regenerating 5th primary remex of a one-year-old chicken

A: Photograph of regenerating remex, with red box highlighting enlarged view of sampled developing region within the follicle. Yellow lines indicate positions of histological sections shown in B–F. B–D: H&E staining showing histological features of barb ridges at different levels of the regenerating feather. E, F: BrdU staining showing patterns of cell proliferation at corresponding levels. Yellow boxes mark areas shown in respective close-up images. Silhouettes on the right depict corresponding development of the barb cortex (blue) and medulla (yellow). Scale bars: 2 mm for A; 1 mm for B; 0.2 mm for C and E; 0.1 mm for D and F. Images were taken by Y.Y. Zhang.

cells right before the incorporation of barbs into the rachis (Figure 5F, 11Biii). As a result, the split barb rami derived from adjacent ridges were not physically separated at their bases, leading to the fusion between two ramus branches before incorporation into the rachis (Figure 5F, 11Biii). The developmental progression of morphotype B feathers may be inferred from a temporal sequence involving: (i) keratinization of basilar cells without further proliferation or differentiation, forming a solid distal beam (Figure 11Biii; tp1); (ii) lateral expansion of the dorsal cortex in the absence of medulla

formation or keratinization (Figure 11Biii; tp2); (iii) midline bifurcation of the expanded dorsal cortex due to the apoptosis of the further centrally extended axial plate (Figure 11Biii; tp3); and (iv) proximal fusion of each split barb ramus branch with the adjacent barb ramus prior to incorporation into the rachis result from the failure of the marginal plate to reach the apical basilar cells (Figure 11Biii; tp4).

In morphotype C, no medullary tissue was identified in the barb rami, suggesting a complete absence of medullary cell differentiation during barb ridge morphogenesis. Unlike the

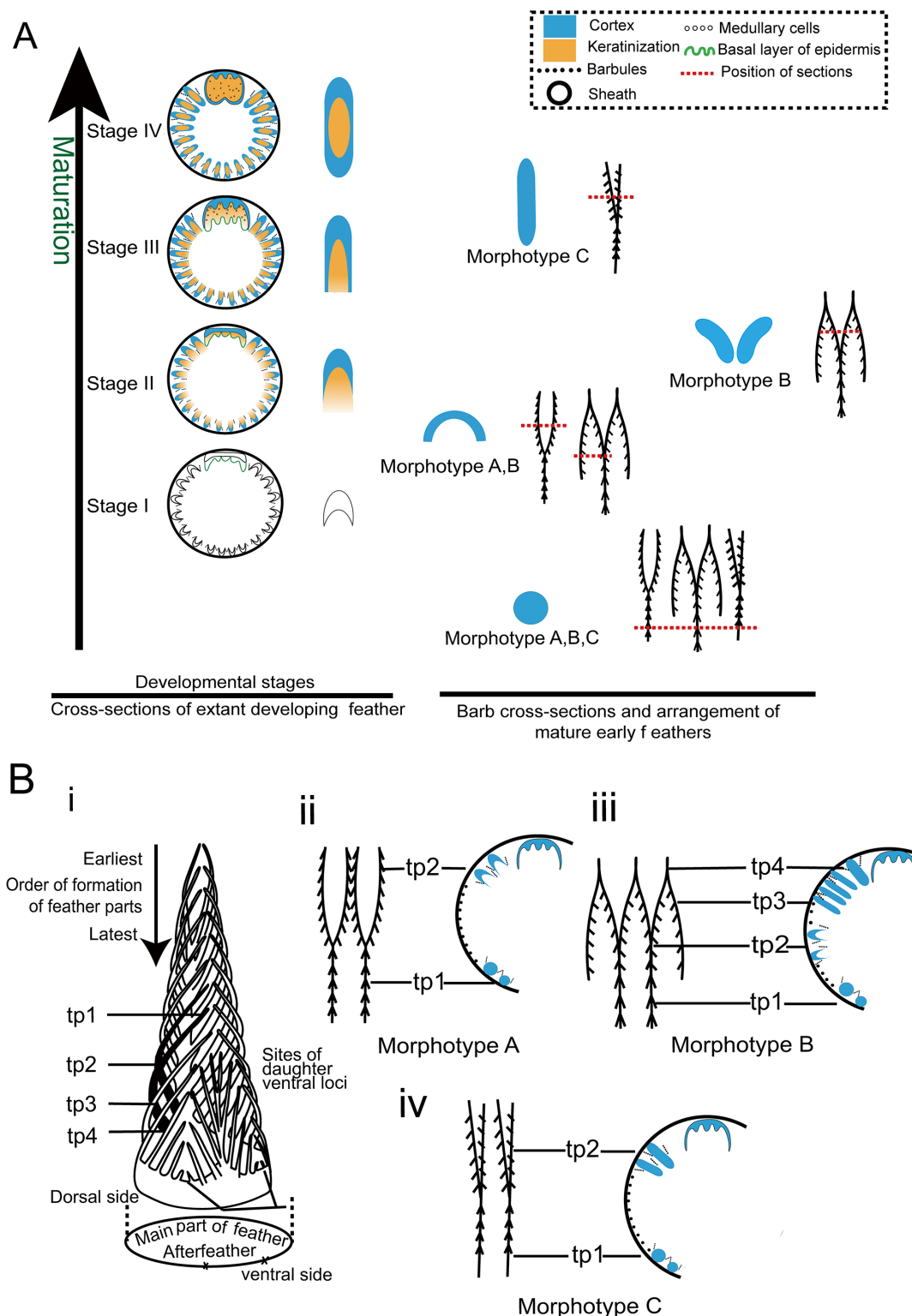


Figure 11 Schematic representation of diverse barb morphologies resulting from incompletely developed barb rami

A: Horizontal cross-sections of a developing extant flight feather (left), demonstrating morphological changes in barb ridges during maturation, alongside corresponding barb rami observed in Cretaceous feathers (right). Comparison highlights how diverse barb morphologies in Cretaceous feathers correspond to various stages of incomplete development in extant feathers. Red dashed lines indicate planes from which cross-sectional silhouettes were taken. B: Schematic of temporal developmental shifts along barb rami across different early feather morphotypes. I: Model of a growing feather (sheath and pulp omitted) showing barb arrangement within the feather follicle (modified from Lucas & Stettenheim (1972)). Black-highlighted barbs correspond to those illustrated in (ii–iv), emphasizing that distal barb tips form first at the new barb locus, and continue elongating proximally toward the rachis. ii–iv: Morphological variations along barb rami (left) resulting from temporal developmental shifts (right) for each feather morphotype. Cross-sections illustrate relative positions of barb ridges at specific time points (tp 1–4) when the corresponding section of the barb rami was formed; however, not all depicted barb ridge morphologies are necessarily present within a single cross-section at any given time point.

transverse expansion observed in ribbon-like rami of morphotype A, morphotype C rami expand dorsoventrally (Figure 11Biv). This suggests that barb rami are fully keratinized to the ventral apex, possibly reflecting a more advanced developmental trajectory compared to the solid beam and ventrally open barb rami of morphotypes A and B.

Several regulatory genes implicated in feather morphogenesis have been identified within the well-established framework of feather development (Chang et al., 2019; Harris et al., 2002; Yu et al., 2002). For example, feather regeneration relies on the collar bulge region (Yue et al., 2005), while a *Wnt3a* signaling gradient oriented from the rachis toward the new barb locus (equivalent to the anterior-posterior axis defined by Prum (1999)) contributes to vane formation in flight feathers but not in down feathers (Yue et al., 2006). *Wnt* inhibitors are also involved in the regulation of feather regeneration and axial patterning (Chu et al., 2014). The arrangement of barb ridges appears to be modulated by *Ephrin B1*, which is expressed in the marginal plate epithelium (Suksaweang et al., 2012). Rachis and the barb growth zone topologies are primarily controlled by *GDF10* and *GREM1*, with retinoic acid signaling playing a critical role in establishing feather asymmetry (Li et al., 2017). Sonic hedgehog (*Shh*) and *Wnt3a* are required for the specification of marginal and axial plate cells, respectively (Chang et al., 2019; Harris et al., 2002; Yu et al., 2002). Although *Bmp4* and *Bmp2* are essential for the differentiation of barbule plates, *Shh* is minimally expressed in these regions (Yu et al., 2002). *Bmp2* exhibits transient expression in the peripheral marginal plates during the early stages of barb ramus development, later switching to the barbule plate epithelia, specifying marginal plate cell fate (Harris et al., 2002). In addition, *MMP2* expression in the marginal plates of adult chicken feather follicles also contributes to this process (Jiang et al., 2011). Experimentally, *Bmp4* overexpression has been shown to increase barb fusion by inhibiting apoptosis in marginal plate cells, while *Shh* suppression produces web-like barb fusions akin to the forked barb rami observed in morphotype B feathers (Yu et al., 2002).

According to the well-established developmental framework (Yu et al., 2002), the interplay between *Noggin* and *Bmp4* regulates barb ridge number, size, and spacing, with *Shh* negatively regulated by *Bmps* (Harris et al., 2002; Yu et al., 2002). Overexpression of *Bmps* suppresses *Shh*, preventing marginal plate formation (Yu et al., 2002). Conversely, down-regulation of *Bmp* promotes branching by enhancing basilar cell ridge-forming activity and facilitating the specification of marginal plate cells (Yu et al., 2002). In addition, *Ski* expression has been implicated with the loss of the medulla in extant feathers (Chang et al., 2019).

Current data suggest that medulla-free barb rami may result from an imbalance between *Bmps* expression and its antagonists (Yu et al., 2002). The bifurcated barb rami characteristic of morphotype B feathers may have originated through heterotopic *Shh* expression at the axial plate and *Wnt3a* at the marginal plates, leading to the reversed positional identities of these molecules at specific developmental stages. These patterns indicate that both heterotopic (spatial) and heterochronic (temporal) alterations in gene expression may have contributed to the formation of split, ventrally open barb rami. In morphotype C feathers, the complete absence of medulla may reflect *Ski*-mediated suppression of medullary differentiation (Chang et al., 2019). Collectively, these observations suggest that diverse

evolutionary developmental mechanisms may account for the atypical barb morphologies observed in early feathers, although these hypotheses await experimental verification as molecular tools advance.

The emergence of ribbon-like barb rami provides three evolutionary insights: (i) Widespread medulla absence in early feathers: The consistent lack of medulla indicates reduced tissue differentiation in both rachis and barbs during early stages of feather evolution. This interpretation is supported by Burmese amber specimens in which the rachis is not appreciably thicker than the barbs. Mechanical strengthening in these structures likely relied on dorsal cortex expansion and increased shaft diameter, leading to ventrally open configurations. However, poor preservation in carbonized fossils limits definitive confirmation; (ii) Developmental plasticity as a source of morphological diversity: The coexistence of medulla-free, ventrally open segments and solid, beam-like sections along a single barb ramus point to substantial spatiotemporal plasticity in developmental programs. This plasticity likely allowed for considerable variability in feather structure, contributing to the morphological diversity observed in early feathers; and (iii) Delayed emergence of medulla in feather evolution: The consistent presence of medullary structures in both rachises and barb rami appears to be a derived trait, implying that complex feather branching preceded the establishment of differentiated internal tissues.

CONCLUSIONS

The findings of this study suggest that tissue differentiation within the branches of some early feathers was relatively limited. In feathers where the medulla either failed to form or failed to undergo keratinization, structural reinforcement of the rachis and barb rami was achieved through expansion of the dorsal cortex. This produced medulla-free, ventrally open rachises and barbs, configurations rarely observed in modern feathers. The absence of a fully developed medulla and ventral cortex likely conferred increased developmental plasticity to these feather components, enabling the emergence of diverse morphotypes not present in modern birds. Morphological variation in early feathers was shaped not only by branching patterns but also by differences in cortex expansion and the plasticity of medullary tissue development. As the medulla and ventral cortex became more consistently established over evolutionary time, the sandwich-like architecture of modern feather branches stabilized, and the developmental flexibility of rachis and barbs was progressively reduced.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

S.W. designed the project; Y.Y.Z., J.W.T., Y.W., and S.W. performed the experiments; Y.Y.Z., J.W.T., Y.W., and S.W. analyzed the data; S.W., Y.Y.Z., and Y.W. wrote the paper. All authors read and approved the final version of the manuscript.

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