PNAS LETTER

Reply to Machida et al.: Although embryology cannot establish the "Protura-sister", phylogenomics can

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Since the turn of the 21st century, rapid advancements in phylogenomics have significantly deepened our understanding of the tree of life, particularly for ancient divergence. A recent letter to the editor (1) challenges our conclusions by invoking embryological evidence from four hexapod classes, arguing that such evidence does not sufficiently support the "Protura-sister" hypothesis. While we acknowledge the valuable insights provided by Machida et al.'s embryological studies, we contend that overemphasizing such evidence in phylogenetic discussions may be misleading. Morphological assessments should be based on a comprehensive evaluation of diverse lines of evidence.

Protura exhibit anamorphosis, an ancestral trait in the postembryonic development of Arthropoda, which is absent in Collembola, Diplura, and Insecta, representing a synapomorphy for Holomeristoma (2, 3). Moreover, the entognathy observed in three noninsect hexapod lineages (Protura, Collembola, and Diplura) evolved from an exognathous ancestral state, representing a highly derived mouthpart configuration (4). However, the precise timing, reasons, and mechanisms behind this evolutionary transition remain enigmatic. Embryonic developmental patterns and morphological differences seem to support the "Ellipura" hypothesis (1, 4). Yet, when examining structural mouthpart interactions, evidence corroborates the notion of Collembola as the sister group to Diplura (5, 6), aligning with the Entognatha sensu Stummer-Traunfels (7). The previous researchers supported different hypotheses based on morphological characteristics of different aspects, and the relationships among the four hexapod classes could not be clarified based on only one or two evidence from embryological development. That is why we are exploring their evolutionary relationships based on the genome-scale molecular data (8).

Hexapods may have originated and diversified as early as the Ordovician or Silurian periods (9). Beyond the Devonian *Rhyniella praecursor*, most early branches have been extinct, making it challenging to fully reconstruct their evolutionary history. To address this, we conducted a comprehensive phylogenomic analysis using multiple evolutionary models and gene filtering strategies, revealing robust phylogenetic relationships among hexapods and exploring the underlying logic of their morphological evolution within this framework (8). Just as the proposition that hexapods are terrestrial crustaceans was once astonishing (10), it has since been well confirmed by phylogenomic studies and is now widely accepted (11). Thus, our phylogenomic work represents a revolutionary advancement in the study of hexapod evolution, providing a robust framework that will guide future morphological research. Of course, it is still necessary to add more representative groups of three noninsect hexapods in the future and develop more accurate molecular evolution analysis methods.

The three noninsect hexapods are cryptic yet pivotal for comprehending the origin and evolution of hexapods. Our research has breathed fresh life into these ancient lineages and underexplored areas, reinvigorating scholarly interest and opening innovative avenues for exploration. We welcome discussions and collaborations to further explore the intricate evolutionary history of these fascinating organisms.

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