Herpetology Notes 19: 133–137, published on 17 March 2026

First report of direct development in *Raorchestes malipoensis* Huang et al., 2023 from southeast Yunnan under laboratory conditions

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The Rhacophoridae of the Old World are renowned for their remarkable species diversity and reproductive modes. According to Meegaskumbura et al. (2015) and Chen et al. (2020), their reproductive strategies can be categorised into four types: aquatic breeding, terrestrial gel nesting, terrestrial foam nesting, and terrestrial direct development. Direct development refers to a reproductive process that bypasses the free-swimming tadpole stage with embryos developing directly from eggs into juvenile frogs. Species within the genera *Pseudophilautus*, *Philautus*, and *Raorchestes* exhibit this mode of reproduction (Bahir et al., 2005; Bossuyt et al., 2004; Biju and Bossuyt, 2005a). During direct development, embryos develop into juvenile frogs within the eggs, without forming tadpole-specific structures such as tadpole teeth, jaws, and a long-coiled intestine. Instead, they exhibit precocious development of frog-specific structures, such as large eyes, frog jaws, and limbs (Elinson and Del Pino, 2012; Bahir et al., 2005; Yan et al., 2021; Zhang et al., 2022).

Direct development represents a successful evolutionary strategy that eliminates aquatic dependence by severing the requirement for water, thereby removing constraints associated with aquatic habitats and enhancing evolutionary success within lineages and it is particularly favoured by anuran species in typical tropical regions. (Elinson, 2013; Liedtke et al. 2017; Salica et al. 2023). Chen et al. (2020) documented two independent evolutionary origins of direct-developing reproductive modes in the family Rhacophoridae, with both transitions emerging nearly synchronously.

The genus *Raorchestes* comprises a total of 78 species, which are distributed from southern India and Nepal eastward to Myanmar, Thailand, Laos, southern China, Vietnam, Cambodia, and West Malaysia (Frost, 2025). Research on direct development in *Raorchestes* has primarily focused on species from the Western Ghats in India, while species outside of India have received less attention (Biju et al., 2010; Bossuyt et al., 2001; Krishnamurthy et al., 2002; Princy and Kannan, 2018; Yan et al., 2021; Zhang et al., 2022). Here, we report on the direct development of a little-known species: the Malipo Bush Frog, *Raorchestes malipoensis* Huang et al., 2023. This species is currently known only from the type locality (Malipo County, Yunnan, China) and Pac Ban, Tuyen Quang, in northern Vietnam, and was initially described based on morphological and molecular evidence (Huang et al., 2023).

Fieldwork was conducted on 17 May 2024 at the type locality of *R. malipoensis* (23.182°N, 104.78°E, elevation 1496 m; Fig. 1). The area is a high-altitude karst cloud forest. The individuals of *R. malipoensis* were located through visual surveys and advertisement calls. Around 20:00 h, we observed a male *Raorchestes* individual emitting advertisement calls near a female on a leaf of *Amomum* sp. (Zingiberaceae; Fig. 2A, B). The female was found near the male, with mature eggs visible ventrally. These two individuals were paired immediately after collection (Fig. 2D). They were identified as *R. malipoensis* based on the morphological match with the original description (Huang et al., 2020), which includes the following characteristics: (1) very small body size (males snout-vent length [SVL] 14.6–17.7 mm, n = 7; females SVL 18.3–19.3 mm, n = 4); (2) head wider than long; (3) small tympanum, supratympanic fold distinct; (4) tips of all fingers and

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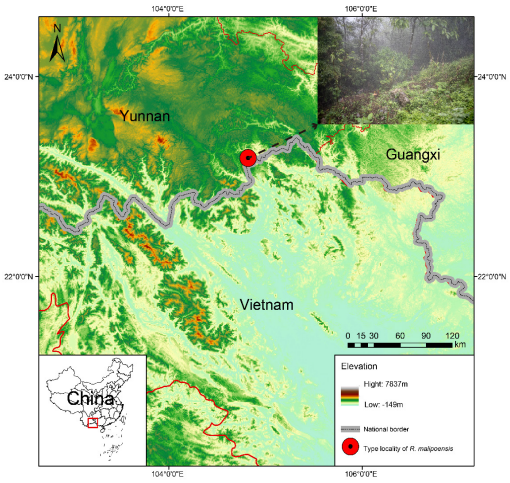

Figure 1. The map of the study area, Malipo, Yunnan, China, the type locality of *R. malipoensis*.

toes yellow; (5) webbing formula (I 2 – 2 II 2 – 2 III 2 – 3 IV 3 – 2 V); (6) inner and outer metacarpal tubercle indistinct; (7) nuptial pad small and milky white.

On 18 May 2024, the female laid 11 eggs beneath the leaf litter, after which both the female and male were released back to their original location. Embryonic developmental stages were monitored under captive conditions. The experimental setup for spawning included mixed vegetation and leaf litter aboveground, maintained at a temperature of 24–26 °C. To maintain humidity for the egg clutches, they were misted with a spray bottle every 3–4 days. The amount of water was controlled to sufficiently moisten the surface of the clutches without causing water pooling on the soil. Natural lighting conditions were maintained throughout the experiment to remain consistent with the natural diurnal rhythm in the wild. Daily observations were recorded to track developmental progression. Egg stages were categorised by comparing them to reference photographs of developmental phases from Bahir et al. (2005), Yan et al. (2021) and Zhang et al. (2022).

The development of the eggs occurred within the vitelline membrane, and fully metamorphosed juveniles eventually emerged from the eggs (Fig. 3N). The

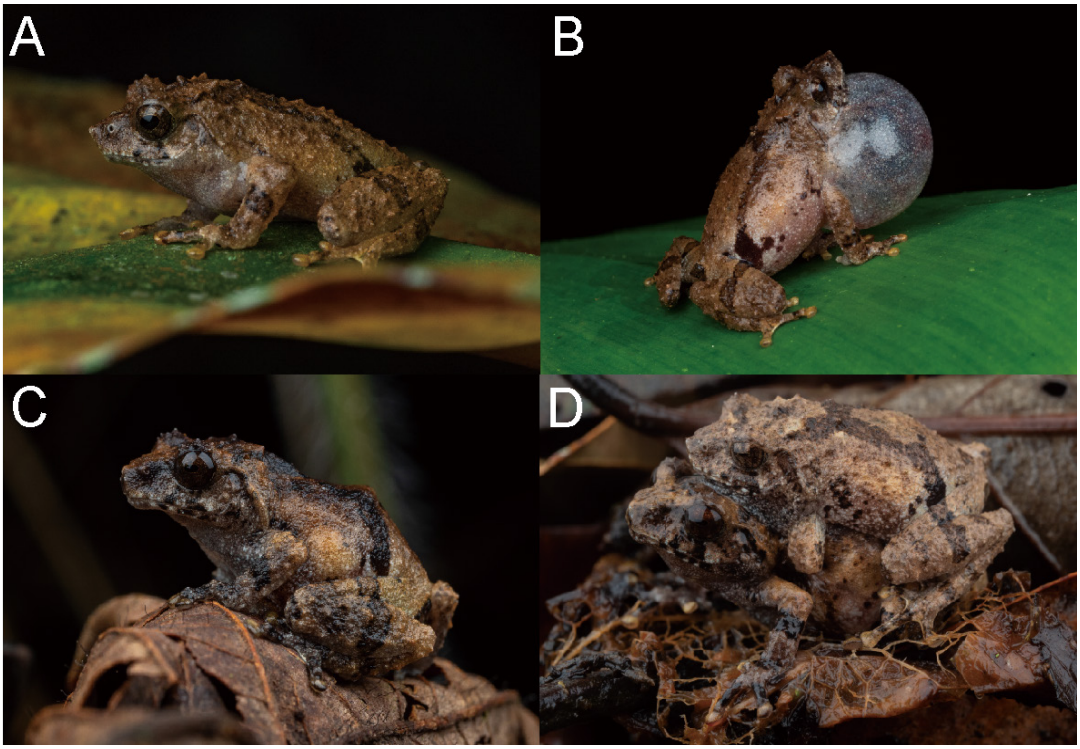

Figure 2. Captured *R. malipoensis* and their behaviours: A) male, B) male emitting advertisement calls, C) gravid female, D) amplexus in captivity. Photos by Junkai Huang.

diameter of the eggs was 3–4 mm (n = 11). During mid-development (12th day), the eggs absorbed water and slightly expanded, compared to newly laid eggs, the vitelline membrane at this stage was tough and elastic but relatively opaque (Fig. 3G), with diameters reaching 7–8 mm (n = 9).

In later developmental stages, the vitelline membrane became softer and appeared colourless (Fig. 3M). Early egg development progressed rapidly (1–19th day). By the 4th day, the head and tail began to differentiate (Fig. 3B). On the 7th day, the formation of eye structures and forelimbs became observable through the vitelline membrane. Development slowed after the forelimbs were fully formed by the 19th day (Fig. 3M). On the 26th day, the froglets began to hatch and were capable of walking, though their tails were still present (Fig. 3N). By the 30th day, the tails of the earliest hatched froglets had almost completely disappeared (Fig. 3O). The hatching process of the froglets could be divided into 15 stages, and based on the relevant characteristics of each stage, we compiled an embryonic development staging table for this species (Table 1; Fig. 3).

Current research on the direct development of terrestrial *Raorchestes* has mainly focused on Western Ghats species (Biju et al., 2010; Bossuyt et al., 2001; Krishnamurthy et al., 2002; Princy and Kannan, 2018). Among Indochinese species (Southwest China, Myanmar, Thailand, Laos, Vietnam, Cambodia, and West Malaysia), only *R. longchuanensis* and *R. huanglianshan* have had their direct development described.

Based on previous studies, we found that *Raorchestes* species utilise diverse types of breeding microhabitats, Type 1) in ground soil: e.g., *R. ghatei* (Padhye et al., 2013), *R. tinniens* (Princy and Kannan, 2018), *R. graminirupes* (Biju and Bossuyt, 2005b), and *R. resplendens* (Biju et al., 2010); Type 2) in hollow bamboo internodes: e.g. *R. chalazodes* (Seshadri and Bickford, 2018); Type 3) upper side of plant leaves: e.g. *R. glandulosus* (Krishnamurthy et al., 2002); Type 4) in tree holes: e.g. *R. nerostagona* (Biju and Bossuyt, 2005a) and *R. rezakhani* (Siammawii et al., 2024); Type 5) tree trunk: e.g., *R. bobingeri* (Biju and Bossuyt, 2005b). Although we were unable to document the oviposition microhabitat during field surveys, observations of oviposition sites under artificial conditions and our field observations documented that its sister species, *R. hekouensis* (Du et al., 2024), deposits eggs beneath the leaf litter formed by *Amomum* sp. (Zingiberaceae) in natural habitats. We hypothesise that this species likely deposits its eggs within the leaf litter in the natural habitats and therefore the breeding microhabitat of *R.*

Table 1. Staging table of embryonic development in *Raorchestes malipoensis*.

Date	Days	Figure	Stage	Developmental Characteristics
18 May 2024	1	3A	1	Fertilised eggs
21 May 2024	4	3B	2	Head and tail ends become demarcated
23 May 2024	6	3C	3	Slight grey colouration over brain region; neural tube closed; hind-limb buds and tail buds visible
24 May 2024	7	3D	4	Unpigmented subdermal eyes visible; head lengthens, slightly further forward beyond yolk; forelimb buds visible; hind limb buds longer than forelimbs; tail about twice its length at Stage 2, laterally curved left or right
25 May 2024	8	3E	5	Dorsal and eyes pigmentation develop; forelimb buds become more prominent adjacent to the head; tail length significantly exceeds the yolk radius
27 May 2024	10	3F	6	Pigmentation dense on the head, snout, and dorsum; forelimb buds longer; eyes colour darker; toe 4 and 5 demarcated
29 May 2024	12	3G	7	Pigmented on ventral side; toe 3 demarcated; cornea clearing, iris black and pupil grey
31 May 2024	14	3H	8	Pigmentation dense on limbs, feet and tail; hindlimbs longer; toes 1 and 2 demarcated; toes 3, 4 and 5 separated
1 June 2024	15	3I	9	Pigmentation dense on ventral; tips of toes enlarged; toes 1–5 distinctly separated; tail similar length with a diameter of yolk
2 June 2024	16	3J	10	Forelimb elbow emerges; eyes larger; legs lengthen
3 June 2024	17	3K	11	Sclera appear silvery-white; further development of the forelimbs and hindlimbs; tips of toes enlarged, showing webbing between the toes; tail more developed
5 June 2024	18	3L	12	Forelimbs and hindlimbs more develop, upper eyelid visible
6 June 2024	19	3M	13	Forelimbs fully developed; fingers distinct, pigmentation begins; tail noticeably thinned and shortened
15 June 2024	26	3N	14	Hatched and walking; yolk sac still present; tail reduced to a small protrusion
19 June 2024	30	3O	15	Yolk sac almost disappeared; tail mostly absorbed and disappeared

Figure 3. Developmental stages 1–15 of *R. malipoensis*. Photos by Junkai Huang.

malipoensis should be putatively classified as Type 1.

In this study, we observed that the tail of *R. malipoensis* embryos did not degenerate during the hatching process but was retained until after hatching, disappearing along with the yolk sac. This tail development pattern is similar to that of *R. huanglianshan* and *R. rezakhani* (Zhang et al., 2022; Siammawii et al., 2024), but differing from *R. longchuanensis* where tail degeneration occurs prior to hatching. Therefore, the tail development patterns vary among *Raorchestes* species.

Acknowledgments. We thank Sheng Lin for his help in field work. This work was supported by the funding agencies, for which we are deeply grateful, of the National Natural Science Foundation of China (NSFC32170478, NSFC32370478), the

Fundamental Research Funds for the Central Universities (SWU-KR22014), the Youth Top Talent Program of Chongqing (CQYC 20220510893) to Z.Y.Y. and the Chongqing Innovation Program for Graduate Research (CYS23192) to J.X.S.

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Accepted by Amaël Borzée