

Sperm Morphology of Five *Rhacophorus* (Amphibia: Anura: Rhacophoridae) Species from China

LI MEI QIN^{1,2}, ZHONG HUA ZHENG^{1,*}, JIAN PING JIANG¹, FENG XIE¹ AND YUN MING MO³

¹Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, 610041, China,

²Postgraduate university of Chinese academy of sciences, Beijing, 100049, China,

³Natural History Museum of Guangxi, Nanning, 530023, China.

*Corresponding author E-mail: zhengzh@cib.ac.cn

Abstract.- Sperm shape and size of five species of the genus *Rhacophorus* from China were investigated in the present study. Our results reveal the presence of two possible monophyletic lineages: the first is composed of *R. chenfui*, *R. dugritei* and *R. omeimontis*, has relatively small spermatozoa with a with a coiled head and a thin tail, while the second, composed of *R. mutus* and *R. megacephalus*, have spermatozoa that are longer with a straight head and filiform shape.

Keywords.- Amphibian, Rhacophoridae, *Rhacophorus*, *Polypedates*, spermatozoa, morphology.

Introduction

The genus *Rhacophorus* (*sensu lato*) is a member of the family Rhacophoridae and contains approximately 80 species worldwide, 24 of which are found in China (Fei et al., 2005). Rhacophorids are predominantly arboreal, sharing with basal ranids expanded digital pads and mantellids the intercalary phalangeal elements (Frost et al., 2006). These frogs are distributed in the tropical and sub-tropical regions of eastern and southern Asia; in China, they inhabit southern areas north to Qinling. Phylogenetic and taxonomic relationships within this genus are still conjectural and controversial. For instance, the genus *Polypedates* is valid according to Frost et al. (2007), but it is absent in Fei et al. (2005). Furthermore, Frost et al. (2007) placed *R. chenfui*, *R. dugritei*, *R. omeimontis* in *Rhacophorus*, while *R. mutus* and *R. megacephalus* were placed in the genus *Polypedates* by Fei et al. (2005).

Previous studies have revealed that some morphological characters of the spermatozoa were unique to given taxa in the Anura (Kuramoto and Joshy, 2000; Kuramoto, 1996; Zheng et al., 2000a, 2000b, 2002). For example, the spermatozoa of most *Rana* species are characterized by a cylindrical head and a thin tail, while spiral and corkscrew-shaped sperm head and waved tail are typical of spermatozoa in the family Megophryidae (Zheng et al., 2002), and fusiform-shaped spermatozoa are unique to the family Bombinatoridae (Zheng et al., 2000a). These studies make it evident that sperm morphology is not only variable between taxa, but can be useful for elucidating taxonomic relationships (Kuramoto and Joshy, 2000). In this study, the shape and size of spermatozoa in *Rhacophorus chenfui*, *R.*

dugritei, *R. omeimontis*, *R. megacephalus* and *R. mutus* were examined for the purpose of resolving their cryptic phylogenetic relationships.

Materials and Methods

Collecting localities are listed in Table 1. All specimens were collected during their breeding seasons, (i.e., May to July) from 1998 to 2005.

Frogs were euthanized by inserting a medical needle through the occipital ostium to destroy the spinal cord. Next, the testes were removed and immediately fixed with 10% formaldehyde, squashed and macerated with a clean toothpick; sperm were suspended on slides, air-dried and stained with acid carmine for 40 seconds. Slides were examined on a ZEISS Axioplan2 light microscope (LM). Other testes were fixed with 3% glutaraldehyde for about two hours and centrifuged at 3000 rpm for 30 s; the supernatant was discarded and the pellet rinsed with double distilled water. A drop of the resulting sperm suspension was placed on a cover slip, air-dried, coated with gold, and observed on a JEOL JSM-5900LV scanning electron microscope (SEM).

Spermatozoan pictures were shot by ZEISS AxioVision 4.0. Sperm length was measured by software ArcView GIS 3.2. Sperm length data were analyzed with SPSS 11.5. The length of sperm head of *Rhacophorus chenfui*, *R. dugritei*, *R. omeimontis* were calculated by the formula $L=Nl\pi$ (L : the length of sperm head, $\pi = 3.14$, l = the diameter of helix, N = the number of turns in the helix).

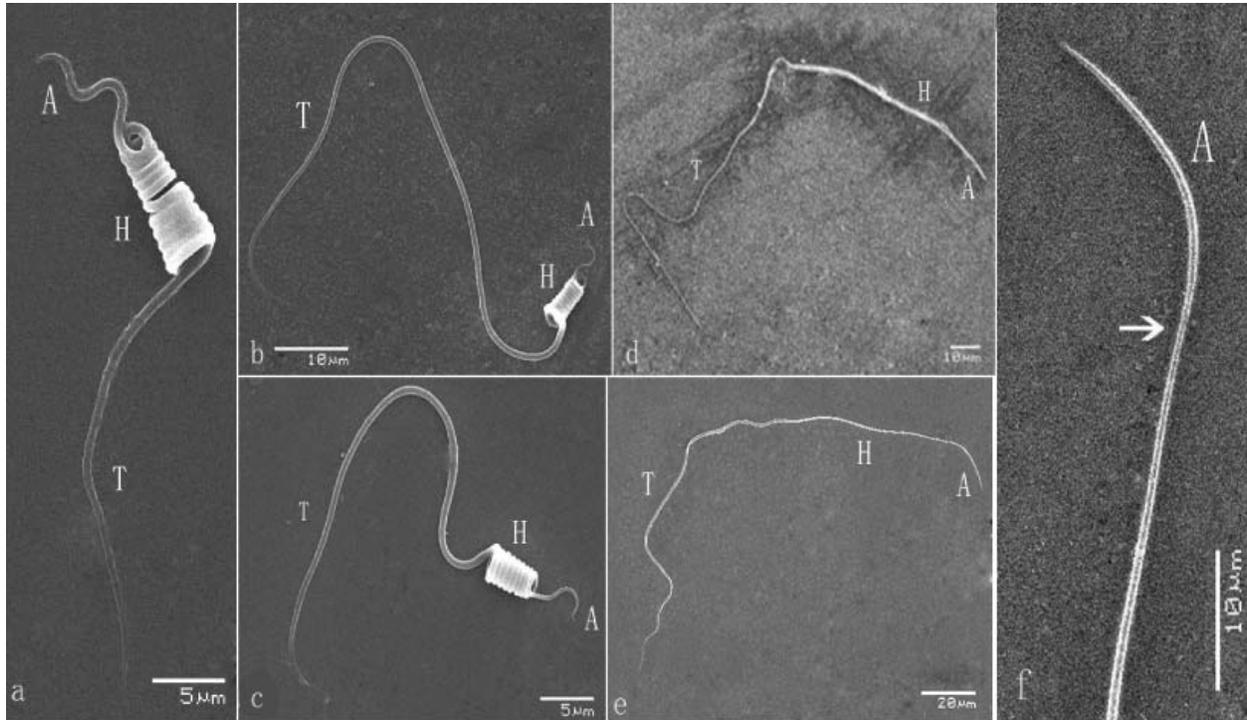


Figure 1. Spermatozoa of five tree frogs. a: *Rhacophorus chenfui*; b: *R. dugritei*; c: *R. omeimontis*; d: *R. mutus*; e: *R. megacephalus*; f: detail of the sperm head of *R. mutus*. A: acrosome; H: head; T: tail.

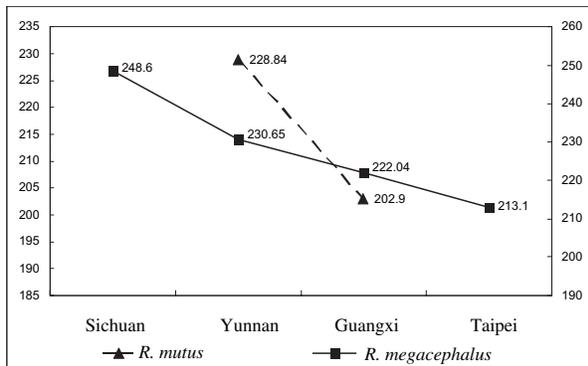


Figure 2. The total length of spermatozoa in different populations of *Rhacophorus mutus* and *R. megacephalus*. Units: μm.

Results

The spermatozoa of all five species consisted of two portions, the head and the tail. The sperm head of *Rhacophorus chenfui* (Fig. 1a) was shaped like a coiled spiral, the tail was thin and wavy (shared with characteristic shared with *R. dugritei* (Fig. 1b) and *R. omeimontis* (Fig. 1c)), and the slightly-coiled apical section was likely the acrosome. The shape of the sperm in *R. mutus* (Fig. 1d) was filiform, consisting of a straight, thick head and a thin wavy tail. A thinner section (Fig. 1f, see

arrow) at the tip of sperm head was likely the acrosome (Fig. 1f, a). Bends and waves could usually be observed from the midpoint of the sperm.

Spermatozoa measurements are shown in Table 1. Total sperm length in *Rhacophorus chenfui* and *R. dugritei* is much shorter than those measured for *R. megacephalus* and *R. mutus*; the sperm of *R. omeimontis* was of intermediate length.

The sperm head of *Rhacophorus dugritei* was the shortest and thinnest, and the head of *R. omeimontis* was longer than that of *R. dugritei*, and thinner than those of all other four species. Head length in *R. chenfui* was also relatively short, and it was also slightly thicker than that of *R. dugritei*. *R. megacephalus* had the largest sperm head among all five species, with the head of *R. mutus* being only slightly smaller.

The coiled head of *Rhacophorus chenfui*, *R. omeimontis* and *R. dugritei* were largely similar. However, the sperm head of *R. chenfui* was longer than the tail, with the ratio of sperm head to total sperm length being 1:0.86.

Sperm size of *Rhacophorus megacephalus* and *R. mutus* differed remarkably between populations. The spermatozoa of *R. megacephalus* in Sichuan population were longer than those in the Guangxi population, and those of *R. mutus* were longer than those in the Yunnan population compared to the Guangxi population (Table 1; Fig. 2).

Table 1. Sperm measurements of *Rhacophorus* species.

Species	Locality	N	Head length	Tail length	Total length	Head width	Turns of head coil
<i>R. chenfui</i>	E'mei,		66.35±7.05	57.10±4.36			
	Sichuan	20	(53.75 %)	(46.25 %)	123.45±8.60	0.76±0.1	8.15±0.59
<i>R. dugritei</i>	Hongya,		59.97±10.67	63.60±16.73			
	Sichuan	20	(47.59 %)	(50.47 %)	126.02±10.97	0.52±0.1	5.90±0.72
<i>R. omeimontis</i>	Hongya,		75.43±8.17	107.99±14.12			
	Sichuan	20	(41.12 %)	(58.88 %)	183.42±17.00	0.50±0.12	6.80±0.62
<i>R. megacephalus</i>	Hejiang,		104.31±12.36	136.85±9.54			
	Sichuan	20	(43.25 %)	(56.75 %)	241.16±11.61	0.89±0.15	—
	Tengchong,		94.05±15.30	136.60±14.38			
Yunnan	20	(40.78 %)	(59.22 %)	230.65±20.20	0.85±0.04	—	
<i>R. mutus</i>	Shangsi,		80.33±12.08	141.71±12.00			
	Guangxi	20	(36.18 %)	(63.82 %)	222.04±11.01	0.83±0.09	—
	Tengchong,		95.32±8.96	133.52±8.66			
Yunnan	20	(41.65 %)	(58.35 %)	228.84±14.01	0.79±0.08	—	
<i>R. mutus</i>	Shangsi,		78.22±10.66	124.68±9.81			
	Guangxi	20	(38.55 %)	(61.45 %)	202.90±11.02	0.83±0.09	—

Unit: μm . N: number of spermatozoa;

Discussion

Spermatozoa morphology.— The morphology of the sperm observed here for *Rhacophorus chenfui*, *R. dugritei* and *R. omeimontis* appeared to be very similar to that reported by Mizuhira et al. (1986) for *R. arboreus* and *R. schlegelii*, as well as that reported by Kuramoto (1996) for *R. viridis amamiensis*, *R. owstoni* and *R. moltrechti*. The sperm heads of latter species, however, while coiled, did not appear to have the two twisted sub-coils characteristic of the former three species. Conversely, the shape of the spermatozoa in *R. mutus* and *R. megacephalus* were very similar to those seen in *Polypedates leucomystax*, *P. megacephalus* (Kuramoto, 1996) and *P. maculatus* (Kuramoto and Joshy, 2000), where the head is linear (not coiled) and no fibers in the tail. Based on the variation of sperm morphology observed here, the five species examined can be divided into two groups: the first group (consisting of *R. chenfui*, *R. dugritei* and *R. omeimontis*) is characterized by a helical sperm head and a thin sperm tail, and the second group (consisting of *R. mutus* and *R. megacephalus*) is characterized by a thread-like sperm head and a thin tail.

Sperm size differ remarkably between species, also between populations. The average length of spermato-

zoa in *Rhacophorus chenfui* was $123.45 \pm 8.60 \mu\text{m}$, while that of *R. megacephalus* was $230.25 \pm 19.81 \mu\text{m}$, a difference of statistical significance ($P < 0.01$, $n = 20$, one-way analysis of variance). In the different populations of *R. megacephalus*, total spermatozoa length in the Sichuan, Yunnan, Guangxi and Taipei populations was $241.16 \mu\text{m}$, $230.65 \mu\text{m}$, $222.04 \mu\text{m}$, $213.1 \mu\text{m}$ (Kuramoto, 1996), respectively, illustrating a reduction in length towards the Pacific Ocean (Fig. 3). This phenomenon was also observed in *R. mutus* (average length $228.84 \mu\text{m}$ in the Yunnan population and $202.9 \mu\text{m}$ in the Guangxi population).

The implications for taxonomy from spermatological data.— On the basis of skeletal characters, Liem (1970) placed *Rhacophorus dugritei* and *R. omeimontis* into the genus *Polypedates*. Uncertain as to the limits of these two genera, Fei (1999) and Fei et al. (2005) tentatively treated all *Polypedates* as *Rhacophorus*. Based on molecular evidence, however, Frost et al. (2007) placed *R. chenfui*, *R. dugritei* and *R. omeimontis* in *Rhacophorus*, and placed *R. mutus* and *R. megacephalus* in *Polypedates*. In view of the spermatological data presented here, the latter hypothesis presented by Frost et al. (2007) appears to be best supported.



Figure 3. Map of collection localities.

Acknowledgments

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