แคริโอไทป์ อิดิโอแกรมมาตรฐาน และการแบ่งเซลล์แบบไมโอซิสของกบนา (*Hoplobatrachus rugulosus*, Dicroglossidae) ด้วยการย้อมสีแบบธรรมดา

Karyotype, Standardized Idiogram and Meiotic Cell Division of Rugose Frog (*Hoplobatrachus rugulosus*, Dicroglossidae) by Conventional Staining

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บทคัดย่อ

การศึกษาแคริโอไทป์และอิดิโอแกรมมาตรฐานของกบนา (*Hoplobatrachus rugulosus*) จากการย้อมสีแบบธรรมดา ใช้ตัวอย่างกบเพศผู้ 5 ตัว และเพศเมีย 5 ตัว จากอำเภอเมือง จังหวัดอุบลราชธานี เตรียมโครโมโซมจากไขกระดูกและอัณฑะ ย้อมสีโครโมโซมด้วยสีจิมซ่า ผลการศึกษาพบว่า กบนามีโครโมโซมดิพลอยด์เท่ากับ 26 แท่ง มีจำนวนโครโมโซมพื้นฐานเท่ากับ 52 โครโมโซมประกอบด้วยเมทาเซนทริกขนาดใหญ่ 2 แท่ง ซับเมทาเซนทริกขนาดใหญ่ 6 แท่ง เมทาเซนทริกขนาดกลาง 2 แท่ง เมทาเซนทริกขนาดเล็ก 4 แท่ง และซับเมทาเซนทริกขนาดเล็ก 12 แท่ง ไม่พบความแตกต่างของโครโมโซมเพศ การแบ่งเซลล์แบบไมโอซิสพบระยะเลปโททีน ไซโกทีน แพคไคทีน ดิโพลทีน ไดอะไคเนซิส เมทาเฟส 1 เมทาเฟส 2 สเปอร์มาทิด และสเปอร์มาโทซัว ระยะเมทาเฟส 1 พบ 13 ไบวาเลนต์วงแหวน ระยะเมทาเฟส 2 มีจำนวนโครโมโซมแฮพลอยด์เท่ากับ 13 แท่ง ข้อมูลที่ได้สามารถนำไปใช้ประโยชน์ทางด้านอนุกรมวิธาน พันธุศาสตร์ วิวัฒนาการ ชีววิทยาการเจริญ การปรับปรุงพันธุ์และการอนุรักษ์ได้

คำสำคัญ: กบนา แคริโอไทป์ อิดิโอแกรม ไมโอซิส

Abstract

The study of standardized karyotype and idiogram of rugose frog (*Hoplobatrachus rugulosus*) by conventional staining, five male and five female frogs of Maung district, Ubon Ratchathani province were used. Chromosomes were prepared from bone marrows and testis followed by Giemsa's staining. The results showed that 2*n*=26 and the fundamental numbers (NF) was 52 in both males and females. The autosomes consist of 2 large metacentric, 6 large submetacentric, 4 medium metacentric, 4 small metacentric and 12 small submetacentric chromosomes. There was no significant difference in sex chromosomes. Meiotic cell division found leptotene, zygotene, pachytene, diplotene, diakinesis, metaphase I, metaphase II, spermatid and

spermatozoa. Metaphase I showed 13 ring bivalents and metaphase II showed haploid number n=13. This information is useful for taxonomy, genetics, evolution, developmental biology, animal breeding and conservation.

Keywords: Rugose frog, Karyotype, Idiogram, Meiosis

Introduction

Rugose frog (*Hoplobatrachus rugulosus*) is known as Chinese edible frog, East Asian bullfrog, or Taiwanese frog. The synonyms of this species are *Rana rugulosa*, *Rana tigrina* ssp. *rugulosa*, *Rana tigrina* ssp. *pantherina* and *H. chinensis*. It is found in Cambodia, China, Laos, Malaysia, Myanmar, Philippines, Thailand and Vietnam. Its natural habitats are freshwater marshes, intermittent freshwater marshes, arable land, pasture land, rural gardens, urban areas, ponds, aquaculture ponds, open excavations, irrigated land, seasonally flooded agricultural land, and canals and ditches. They are widely farmed in Thailand, China and Malaysia. In Thailand, these frogs have been consumed for long time. The rugose frogs are important economic animals, some strange skin frogs (albino or golden) are exotic pets. It is listed in Appendix II of CITES and LC (least concern) in IUCN (Kosuch *et al*., 2001; Chan-ard, 2006; Yu *et al*., 2015).

H. rugulosus is a species in the Dicroglossidae family. The molecular data suggested that this frog may be cryptic species complex including Thai and Chinese populations. The genus *Hoplobatrachus* has five described species, *H. crassus, H. occipitalis, H. rugulosus, H. tigerinus* and *H. litoralis*. The family Dicroglossidae contains more than 186 species in 15 genera in the world. In Thailand, there are 30 species, 7 genera, 2 subfamilies, namely subfamily Dicroglossidae (genera *Nanorana, Fejervarya, Limnonectes, Hoplobatrachus* and *Quasipaa*) and subfamily Occidozyginae (genera *Occidozyga and Ingerana*) (Chan-ard, 2006; Pyron and Wiens, 2011; Hasan *et al*., 2012; Yu *et al*., 2015).

There are several reports on publish cytogenetic studies of *H. rugulosus* including Chang *et al*. (1984), Supaprom and Baimai (2005), Donsakul (2009), Alam *et al*. (2012), Suttichaiya *et al*. (2016) and Tengjaroenkul *et al*. (2017). Previous reports were studied about diploid number, fundamental number (NF, number of chromosome arms), size and shape of chromosomes, karyotype by conventional staining, NOR-banded and C-banded metaphase and chromosome aberration. There are no report standardized karyotype formula, standardized idiogram and meiosis cell division. This research will report diploid number, NF, size and type of chromosomes, standardized karyotype, idiogram and karyotype formula, meiosis cell division, spermatogenesis and spermatozoa character.

Materials and Methods

The study of karyotype, standardized idiogram and meiotic cell division of rugose frog (*Hoplobatrachus rugulosus*, dicroglossidae) by conventional staining was divided into 4 steps including the frog chromosome preparation, chromosome counting, chromosome measuring and chromosome analysis.

1. Frog chromosome preparation: The rugose frog, *Hoplobatrachus rugulosus* (five males and five females) were collected from natural area from Maung district, Ubon Ratchathani, Thailand. The samples were checked by identification key, Chan-ard (2006). Chromosome preparation was conducted by the colchicine-hypotonic-fixation-air drying technique, from bone marrows and testis. The chromosomes were stained with 20% Giemsa's for 30 min (Verma and Babu, 1995).

2. Chromosome counting: Many pictures of the chromosomes in metaphase cells were taken for counting the number of chromosomes. Twenty metaphase cells of each male and female were measured for length of short arm (Ls) and long arm (Ll) chromosomes.

3. Chromosome measuring: The Ls and Ll were measured and calculated for the length of total arm chromosomes (LT, LT=Ls+Ll). Relative length (RL), centromeric index (CI) and standard deviation (SD) were also calculated. The CI was also computed to classify the shape of chromosomes, respectively according to Chaiyasut (1989) and Tanomtong (2011). All parameters were used in standardized karyotyping and idiograming.

4. Chromosome analysis: In karyotype formula, classify the size of chromosome, which the symbol "L, M and S" represent large, medium and small chromosomes, respectively. Classify the type of the chromosome, which the symbol "m, sm, a and t" represent metacentric, submetacentric, acrocentric and telocentric chromosomes, respectively. The NF was obtained by assigning a value of two to metacentric, submetacentric and acrocentric chromosomes and one to telocentric chromosome.

Results

The general character of *H. rugulosus* is showed on Figure 1. The dorsal body is olive-brown or gray with distinct ridges. Ventral is white, sometimes with dark spotting. Front legs have four fingers and hind legs have fully webbed five fingers. Males have a pair of gray subgular vocal sac on the underside of the jaw angle but females lack this vocal sac. The karyotype showed that the number of diploid chromosome of *H. rugulosus* was 2*n*=26 consisting of metacentric and submetacentric chromosomes, the NF was 52 in both males and females (Figure 2). The chromosome types were

present as 2 large metacentric, 6 large submetacentric, 4 medium metacentric, 4 small metacentric and 12 small submetacentric chromosomes. No heteromorphic sex-chromosome was observed between male and female chromosomes. Twenty metaphase cells of each male and female were measured for Ls, LI, LT, CI, RL, SD, chromosome sizes and types were showed on Table 1. This is the first report of standardized idiogram of *H. rugulosus* (Figure 3). The karyotype formula can be deduced as: $2n$ (26) = $L^{m}_{2} + L^{sm}_{6} + M^{m}_{2} + S^{m}_{4} + S^{sm}_{12}$

The first report of meiosis cell division and spermatogenesis *H. rugulosus* showed interphase, leptotene, zygotene, pachytene, diplotene, diakinesis, metaphase I, metaphase II, spermatid and spermatozoa (Figure 4). The sperm head is very thin and narrow, ranging from about 15-20 μm. During metaphase I (meiosis I, reductional division) the homologous chromosomes revealed synapsis, which can be defined as the 13 ring bivalents (4 ring bivalents of metacentric, 9 ring bivalents of submetacentric chromosomes) and n=13 haploid chromosomes on metaphase II (meiosis II, equational division) (Figure 5).

Discussion

The karyotype of *H. rugulosus* was $2n$=26, which accordance with previous report. The NF was 52 in both males and females, which agree with Chang *et al*. (1984), Supaprom and Baimai (2005), Donsakul (2009), Alam *et al*. (2012), Suttichaiya *et al*. (2016) and Tengjaroenkul *et al*. (2017). The autosomes of rugose frog from Maung district, Ubon Ratchathani province were 2 large metacentric, 6 large submetacentric, 4 medium metacentric, 4 small metacentric and 12 small submetacentric chromosomes. The types of autosome were not consistent with Chang *et al*. (1984), Supaprom and Baimai (2005), Donsakul (2009), Suttichaiya *et al*. (2016) and Tengjaroenkul *et al*. (2017). The cause of different type of chromosome may be genetic variation between populations. The frog karyotypes in this study are very similar to Supaprom and Baimai (2005) which studied the frog from Khong Chiam district, Ubon Ratchathani. Moreover, the other reports have investigated frogs from the following areas: Bangkok (Donsakul, 2009), Khon Kaen, more apart population may be more different karyotype (Suttichaiya *et al*., 2016 and Tengjaroenkul *et al*., 2017) and Zhejiang, China (Chang *et al*., 1984) (Table 2).

No heteromorphic sex-chromosome was observed in this study. Some reports show the chromosomal sex determination ZZ/ZW of the Indian bullfrog (*H. tigerinus*) (Saba and Tripathi, 2014). In Thailand, the amphibian sex chromosome system was found in *Fejervarya limnocharis* which indicated that this frog was XY/XX system (Patawang *et al*., 2014).

Karyotypic variation of genus *Hoplobatrachus* is showed on Table 2, namely *H. tigerinus* from India, *H. rugulosus* from China and Thailand and hybrid frog (*H. rugulosus* x *H. tigerinus*). The Indian bullfrogs (*H. tigerinus*) were 2*n*=26, NF=52, the karyotypes comprised metacentric and submetacentric chromosome. The *H. rugulosus* were 2*n*=26, NF=52, the karyotypes comprised metacentric and submetacentric chromosome too. Interestingly, the interspecific hybrid frog between male *H. rugulosus* and female *H. tigerinus* showed the triploid number 3*n*(3x)=39 (Yadav and Pillai, 1975; Chakrabarti *et al*., 1983; Chang *et al*., 1984; Supaprom and Baimai, 2005, Donsakul, 2009, Alam *at al*., 2012; Saba and Tripathi, 2014; Suttichaiya *et al*., 2016 and Tengjaroenkul *et al*., 2017). The other frog in genus *Hoplobatrachus*, *H. crassus, H. occipitalis,* and *H. litoralis* also has no cytogenetic report. This study is useful for taxonomy, genetics, evolution, developmental biology, animal breeding and conservation.

There are two reports of meiosis cell division and spermatogenesis of genus *Rana* in Thailand were studied by Chavadej et al. (2000) and Manochantr *et al*. (2003) which showed in the *R. catesbeiana* and *R. tigerina*, respectively. The results showed all stages of meiosis cell division and spermatozoa morphology. The sperm head was similar to *R. rugulosus* which has highly elongate and cylindrical shape, ranging from about 10-20 μm. However, the previous reports did not show meiotic configuration and haploid karyotype.

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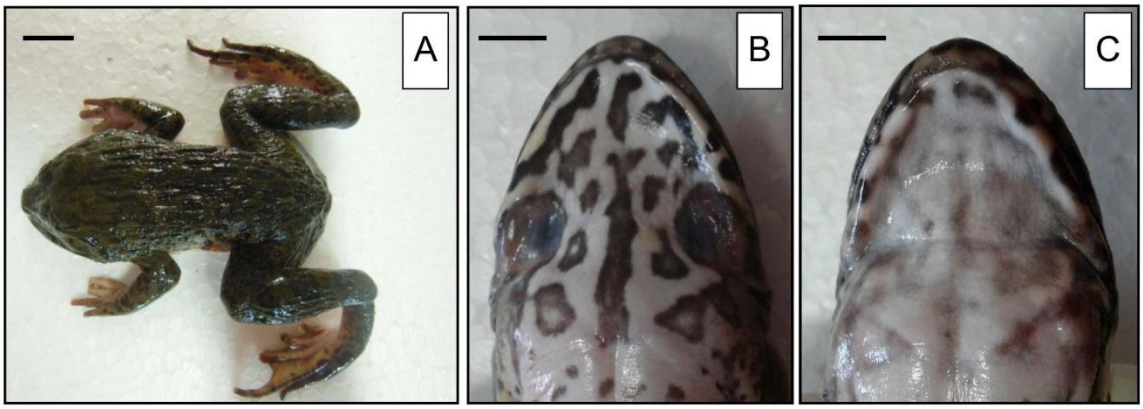

Figure 1 Dorsal view (A), ventral of male (B) and female (C) rugose frog, *Hoplobatrachus rugulosus* (Dicroglossidae, Anura) from Maung district, Ubon Ratchathani (Scale bars = 1 cm).

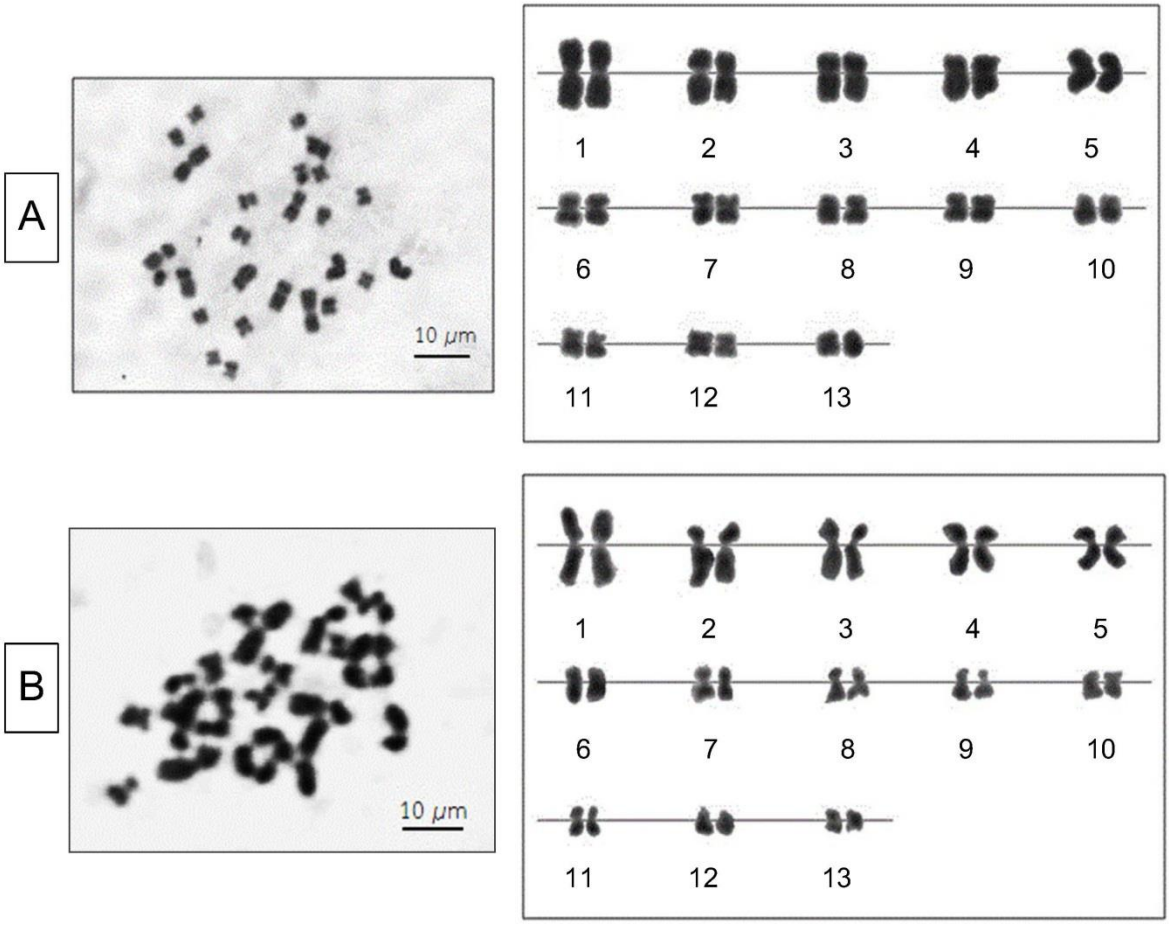

Figure 2 Metaphase plates and standardized karyotypes of male (A) and female (B) rugose frogs, (*Hoplobatrachus rugulosus*) 2*n*=26 by conventional staining (Scale bars = 10 μm).

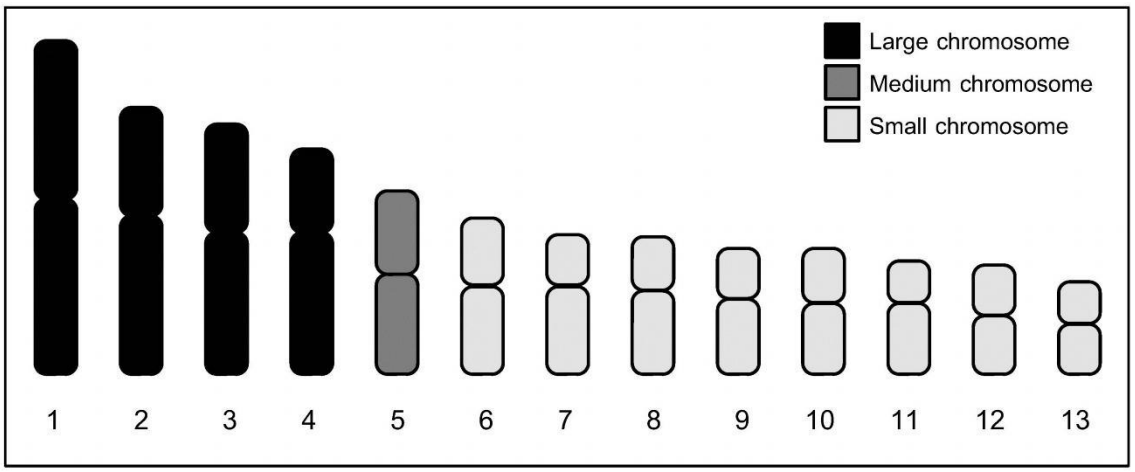

Figure 3 Standardized idiogram of the rugose frog, (*Hoplobatrachus rugulosus*) 2*n*=26 by conventional staining.

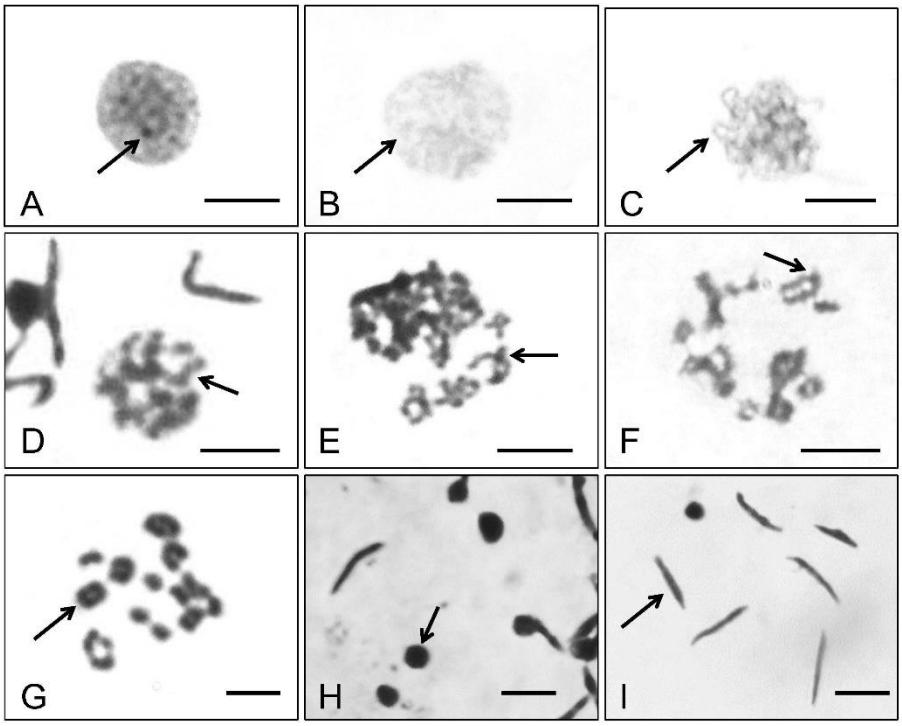

Figure 4 Meiotic cell division of the rugose frog, (*Hoplobatrachus rugulosus*) on interphase (A, arrow indicate nucleolus), leptotene (B, arrow indicate chromatin), zygotene (C, arrow indicate synapsis), pachytene (D, arrow indicate chromatin), diplotene (E, arrow indicate chiasma), diakinesis (F, arrow indicate terminalization), metaphase I (G, arrow indicate ring bivalent), spermatid (H, arrow) and spermatozoa (I, arrow) (Scale bars = 10 μm).

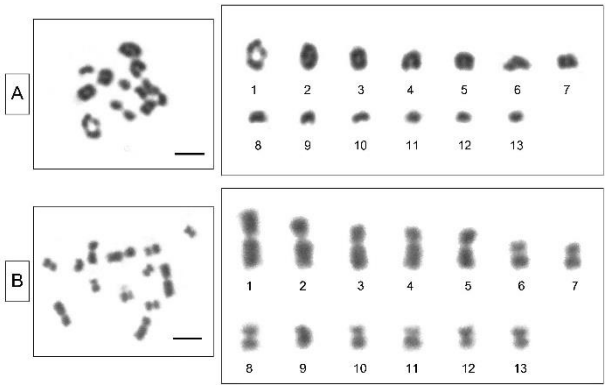

Figure 5 Metaphase I plate and karyotype, 2*n*=26 (A), metaphase II plate and karyotype, *n*=13 (B) of rugose frog, (*Hoplobatrachus rugulosus*) by conventional staining (Scale bars = 10 µm).

Table 1 Mean length of short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), centromeric index (CI), relative length (RL) and standard deviation (SD) of CI, RL from 40 karyotypes of male and female rugose frog, (*Hoplobatrachus rugulosus*), 2*n*=26.

Chromosome pair	Ls (µm)	Ll (µm)	LT (µm)	CI±SD	RL±SD	Chromosome size	Chromosome type
1	4.70	5.71	10.41	0.549±0.021	0.148±0.015	Large	Metacentric
2	3.28	5.01	8.28	0.605±0.006	0.118±0.006	Large	Submetacentric
3	3.05	4.74	7.79	0.608±0.015	0.110±0.010	Large	Submetacentric
4	2.74	4.31	7.04	0.612±0.015	0.100±0.008	Large	Submetacentric
5	2.56	3.40	5.95	0.571±0.022	0.085±0.005	Medium	Metacentric
6	1.92	2.86	4.78	0.602±0.003	0.068±0.005	Small	Submetacentric
7	1.79	2.75	4.54	0.606±0.011	0.065±0.004	Small	Submetacentric
8	1.66	2.56	4.22	0.607±0.006	0.060±0.005	Small	Submetacentric
9	1.55	2.40	3.94	0.609±0.007	0.056±0.005	Small	Submetacentric
10	1.46	2.26	3.72	0.608±0.015	0.053±0.006	Small	Submetacentric
11	1.35	2.10	3.45	0.609±0.008	0.049±0.005	Small	Submetacentric
12	1.38	1.83	3.21	0.570±0.024	0.046±0.005	Small	Metacentric
13	1.27	1.65	2.92	0.565±0.021	0.042±0.004	Small	Metacentric

Table 2 Cytogenetic publications of the genus *Hoplobatrachus*.

Species	Population	2*n*	NF	Chromosome type		Reference
				m	sm	
H. tigerinus	India	26	52	-	-	Yadav and Pillai (1975)
	India	26	52	-	-	Chakrabarti *et al*. (1983)
	India	26	52	16	10	Saba and Tripathi (2014)
	India	26	-	-	-	Alam *et al*. (2012)
H. rugulosus	China	26	52	18	8	Chang *et al*. (1984)
	Thailand	26	52	12	14	Supaprom and Baimai (2005)
	Thailand	26	52	20	6	Donsakul (2009)
	Thailand	26	-	-	-	Alam *et al*. (2012)
	Thailand	26	52	8	18	Suttichaiya *et al*. (2016)
	Thailand	26	52	8	18	Tengjaroenkul *et al*. (2017)
	Thailand	26	52	10	16	This study
Hybrid frog (*H. rugulosus* x *H. tigerinus*)	Thailand x India	39 (3*n*)	-	-	-	Alam *et al*. (2012)

Remarks: 2*n* = diploid chromosome, 3*n* = triploid number, NF = fundamental number, m = metacentric chromosome, sm = submetacentric chromosome, and - = not available.