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The Identification of the Sex Chromosome and Karyotype of Four Toad Species (Genus *Bufo*) in Thailand by T-lymphocyte Cell Culture

Pornnarong Siripiyasing[1], Warawut Chulalaksananukul[2], Putsatee Pariyanonth[3], Sarawut Kaewsri[4], Sarawut Sittigul[5], Namphunk Seatung[6] and Alongkoad Tanomtong[6,*]

[1] Major of Biology, Faculty of Science and Technology, Mahasarakham Rajabhat University, Muang, Mahasarakham 44000, Thailand
[2] Department of Botany, Department of Biology, Faculty of Science, Chulalongkorn University, Phaya-Thai, Bangkok, 10330, Thailand
[3] Department of Biology, Faculty of Science, Chulalongkorn University, Phaya-Thai, Bangkok, 10330, Thailand
[4] Program in Applied Biology, Department of Science, Faculty of Science, Buriram Rajabhat University, Muang, Buriram 31000, Thailand
[5] Program in Biology, Department of Science and technology, Faculty of Science, Pibulsongkram Rajabhat University, Muang, Phitsanuloke 65000, Thailand
[6] Department of Biology, Faculty of Science, Khon Kaen University, Muang, Khon Kaen 40002, Thailand

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Summary Our knowledge is the first report on karyotypic study of four toad species (genus *Bufo*) in Thailand namely; large-eared toad (*Bufo macrotis* Boulenger 1887), Indochinese dwarf toad (*Bufo parvus* Boulenger 1887), common Indian toad (*Bufo melanostictus* Schneider 1799) and river giant toad (*Bufo asper* Gravenhorst 1829). Blood samples were taken from 5 males and 5 females of each four toad species. After the standard whole blood T-lymphocyte culture in the presence of colchicine, the metaphase spreads were performed on microscopic slides and air-dried. Conventional staining, G-banding and C-banding techniques were applied to stain the chromosomes. The results indicated $2n=22$ and fundamental number (NF) 44 in both male and female of four toad species. The autosomes of *B. macrotis* and *B. melanostictus* is being as 18 metacentric and 4 submetacentric chromosomes while *B. parvus* and *B. asper* is as 16 metacentric and 6 submetacentric chromosomes. G-banding technique showed a *B. melanostictus*'s constriction on short arm of Y chromosome (the largest chromosome) but did not show on X chromosome. C-banding technique demonstrated a dark band constriction on Y chromosome of *B. melanostictus*, the representative of constitutive heterochromatin. However, there is no dark band constriction on X chromosome. So, we conclude that the sex determination of *B. melanostictus* is XY system. Although we do not treat *B. macrotis*, *B. parvus* and *B. asper* with G-banding and C-banding technique, we also predict that those three species have the same sex determination as *B. melanostictus*. We extremely appreciate to public our present research, the first cytogenetic study of *B. macrotis* and *B. asper*.

Key words Chromosome, Large-eared toad (*Bufo macrotis*), River giant toad (*Bufo asper*), Indochinese dwarf toad (*Bufo parvus*), Common Indian toad (*Bufo melanostictus*)

General amphibians are belonging to the phylum Chordata, class Amphibia, subclass Lissamphibia and three orders as following: order Gymonphina (Apoda), Caudata (Urodela) and Anura (Salientia) (Duellman 1982). Amphibians in the family Bufonidae (toad) in Thailand have 4 genera including *Ansonia*, *Leptophryne*, *Pedostibes* and *Bufo*. There are 4 species in genus *Bufo*. In this re-

* Corresponding author, e-mail: tanomtong@hotmail.com

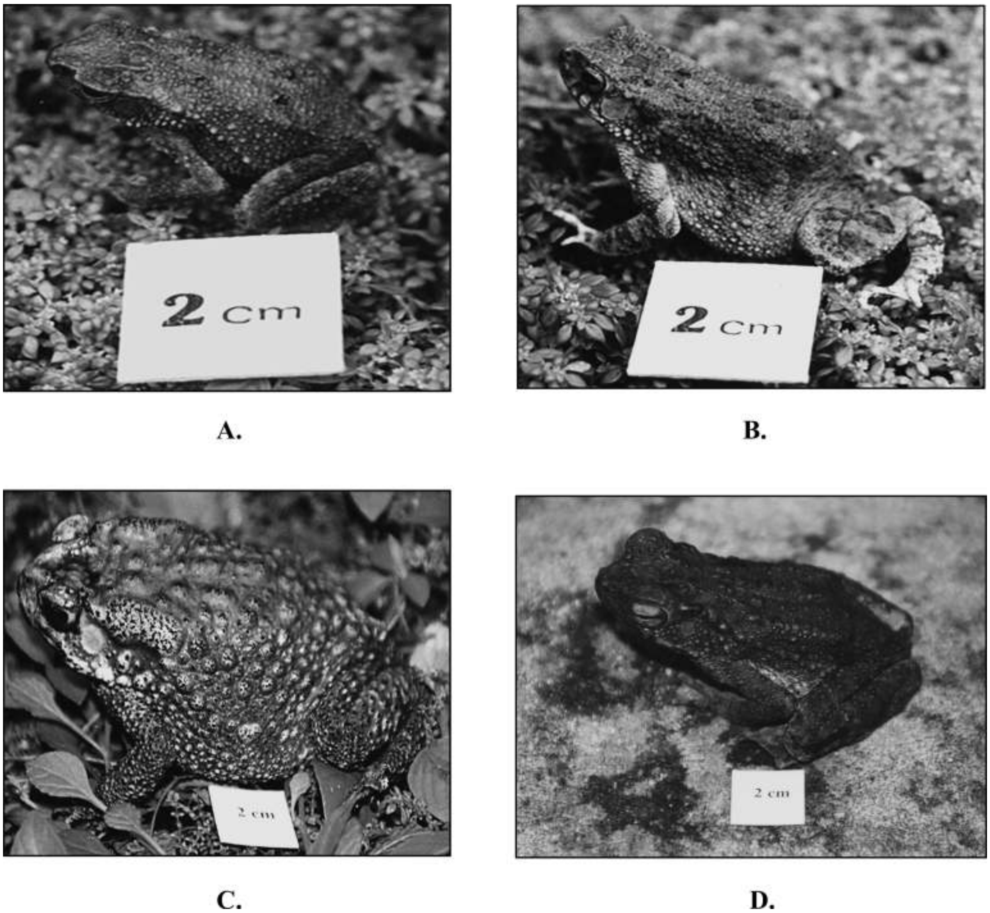
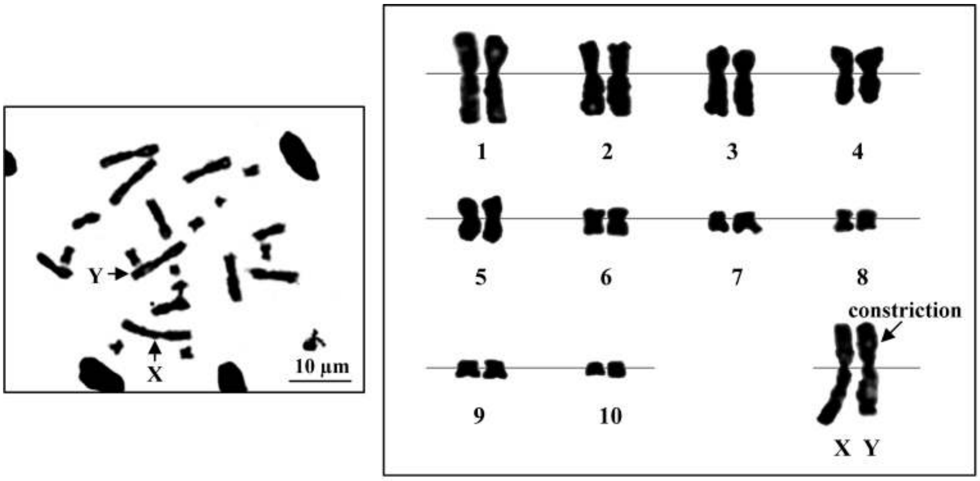


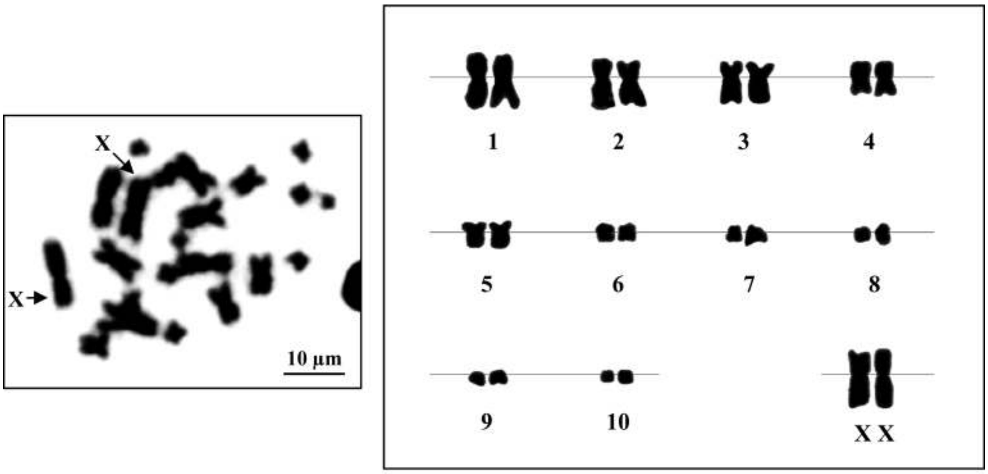
Fig. 1. Four toad species of the genus *Bufo* in Thailand namely; large-eared toad, *Bufo macrotis* (A) Indochinese dwarf toad, *Bufo parvus* (B) common Indian toad, *Bufo melanostictus* (C) and river giant toad, *Bufo asper* (D). Photograph: Pornnarong Siripiyasing and Alongkoad Tanomtong.

search, we have studied all species of the genus *Bufo* including large-eared toad (*Bufo macrotis* Boulenger 1887), Indochinese dwarf toad (*Bufo parvus* Boulenger 1887), common Indian toad (*Bufo melanostictus* Schneider 1799) and river giant toad (*Bufo asper* Gravenhorst 1829) (Fig. 1). The feature characteristic of genus *Bufo* are an oval and untwines elongate tongue, no vomerine tooth, no web on toes, one vocal sac, horizontal oval pupil and obviously parotid gland (Taylor 1962).

In early nineteenth century knowledge, several scientists accepted that amphibians have no sex-chromosome (Solari 1994). In later stage, banding technique was established for chromosome staining which can identify type of sex-chromosome. Amphibians sex-chromosome staining indicated that some species have similar chromosome type but have difference banding pattern. It revealed that there are some genes involved in sex-determination on the chromosome (Schmid *et al.* 1991). All previous knowledge demonstrated that there are several patterns of sex-chromosomes; a group which have difference type and size of sex-chromosome (Schmid 1980, Schmid *et al.* 1983, 1988, Kuramoto 1980, Mahony 1991, Nishioka *et al.* 1994), a group which have difference banding pattern of sex-chromosome (Iturra and Veloso 1989, Schmid *et al.* 1989, 1990, 1991, 1993), a group which have supernumerary of sex-chromosome (Schmid 1978a, Green 1998) and a group which have complex pattern or have no difference type and size of sex-chromosome (Schmid *et al.*



A. The male common Indian toad (*Bufo melanostictus*)



B. The female common Indian toad (*Bufo melanostictus*)

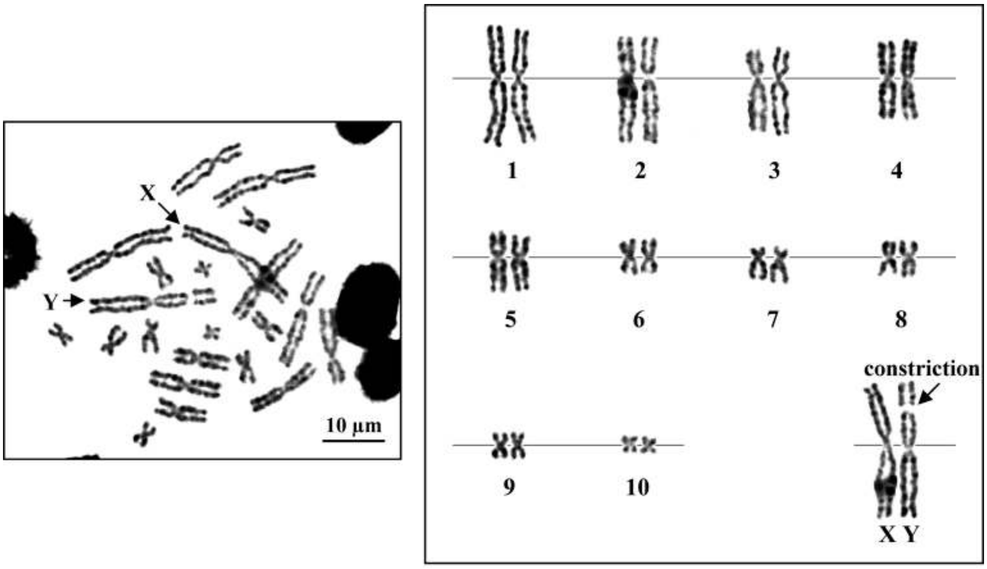
Fig. 2. Metaphase chromosome plates and karyotypes of male (A) and female (B) common Indian toad (*Bufo melanostictus* Schneider 1799) 2*n* (diploid)=22 by conventional staining technique, showing sex chromosomes (arrows).

1978b, 1992, Kuramoto 1980, Schempp and Schmid 1981, Nishioka *et al.* 1987, Melo *et al.* 1995, Ota and Matsui 1995).

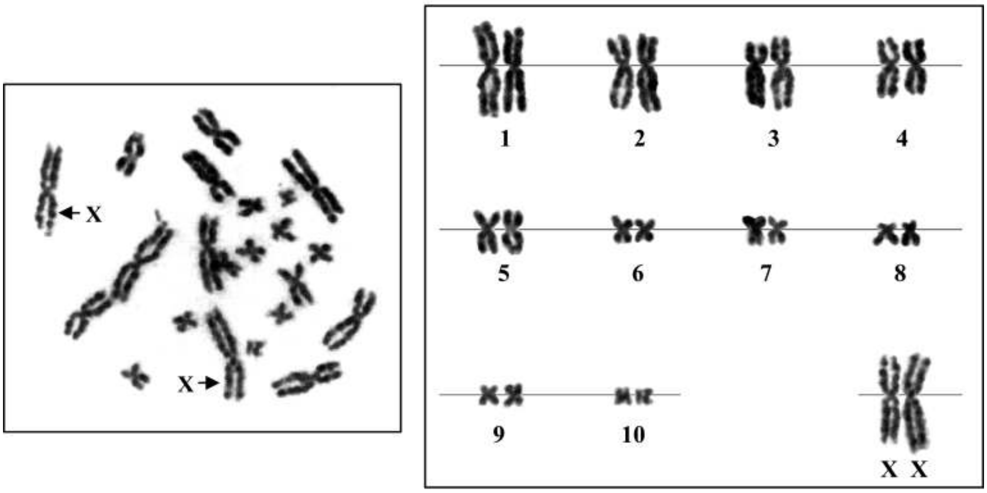
There are numerous cytogenetic reports on toads in genus *Bufo* (Bogart 1966, Beckert and Doyle 1967, Cole *et al.* 1968, Schmid 1978a, 1978b, Narkkasem 1975, Supaporm and Kanlayaprasith 1990). However, there is no report on *B. macrotis* and *B. asper*. This is the first cytogenetic study on those two species as described above.

Materials and methods

Blood samples of 5 males and 5 females of each four toad species in Thailand were collected



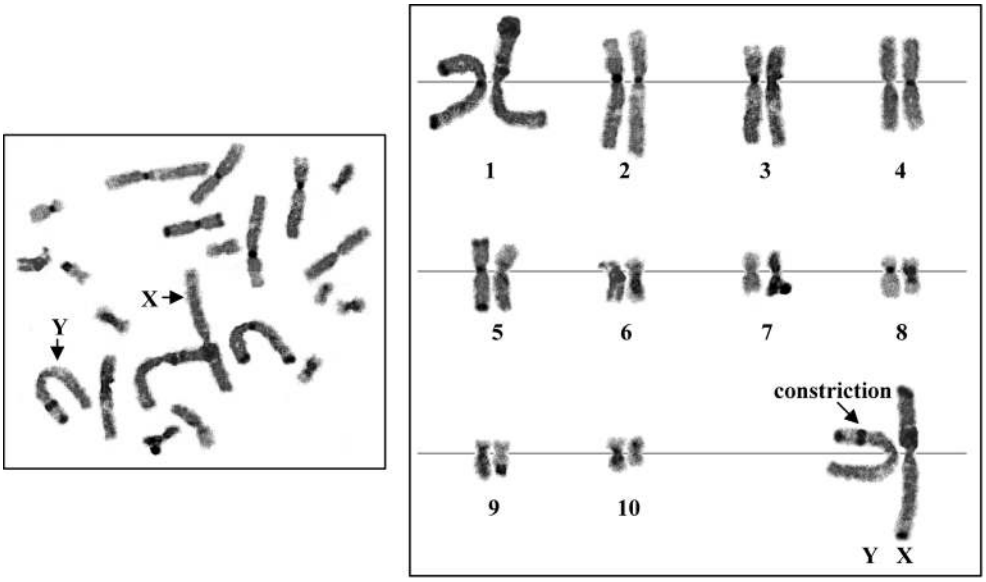
A. The male common Indian toad (*Bufo melanostictus*)



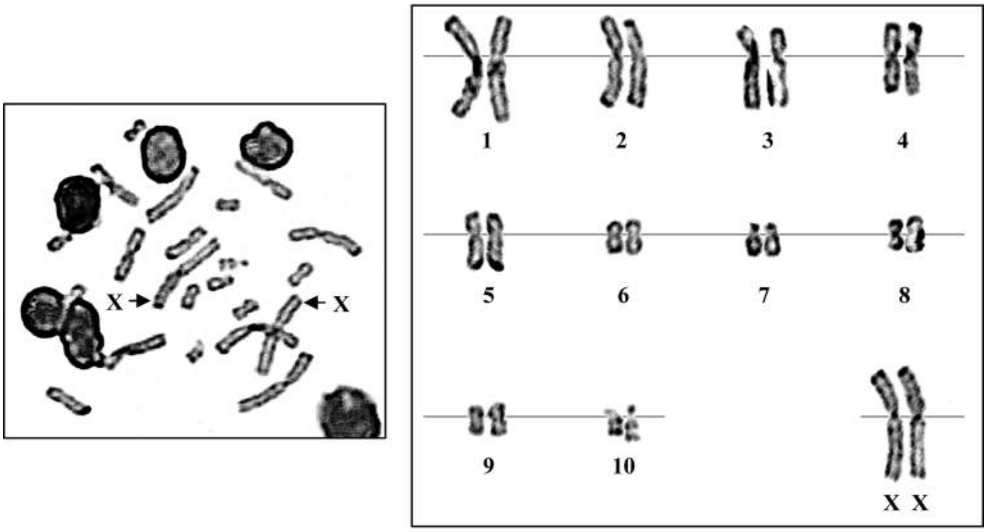
B. The female common Indian toad (*Bufo melanostictus*)

Fig. 3. Metaphase chromosome plates and karyotypes of male (A) and female (B) common Indian toad (*Bufo melanostictus* Schneider 1799) 2*n* (diploid)=22 by G-banding technique, showing sex-chromosomes (arrows).

from Songkla and Kanchanaburi Provinces and then applied for cytogenetic studies by lymphocyte culture of whole blood samples. The culture cells were treated with a colchicine-hypotonic-fixation-air-drying technique followed by conventional staining, G-banding and C-banding techniques with Giemsa's (Rooney 2001, Campiranon 2003). Twenty cells of each individual chromosome checks accomplished by using a light microscope (Chaiyasut 1989).



A. The male common Indian toad (*Bufo melanostictus*)

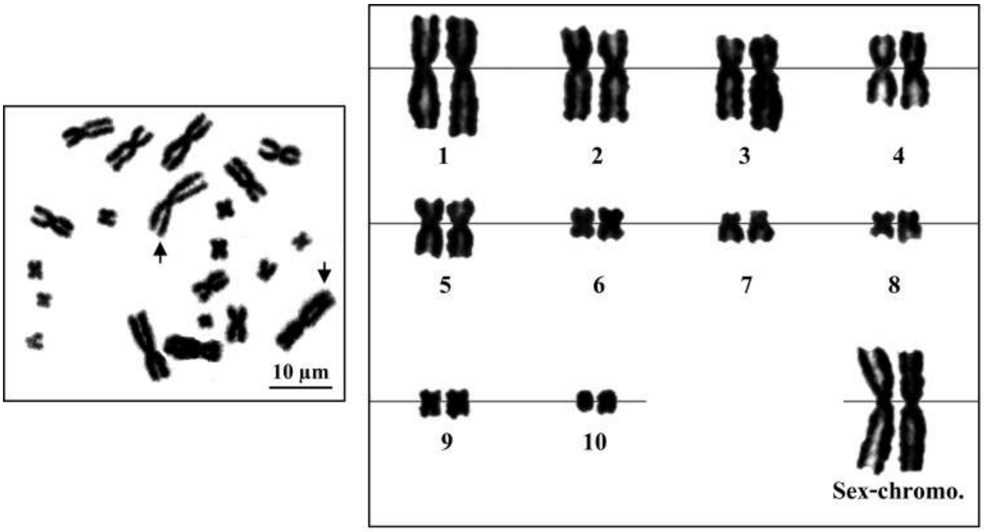


B. The female common Indian toad (*Bufo melanostictus*)

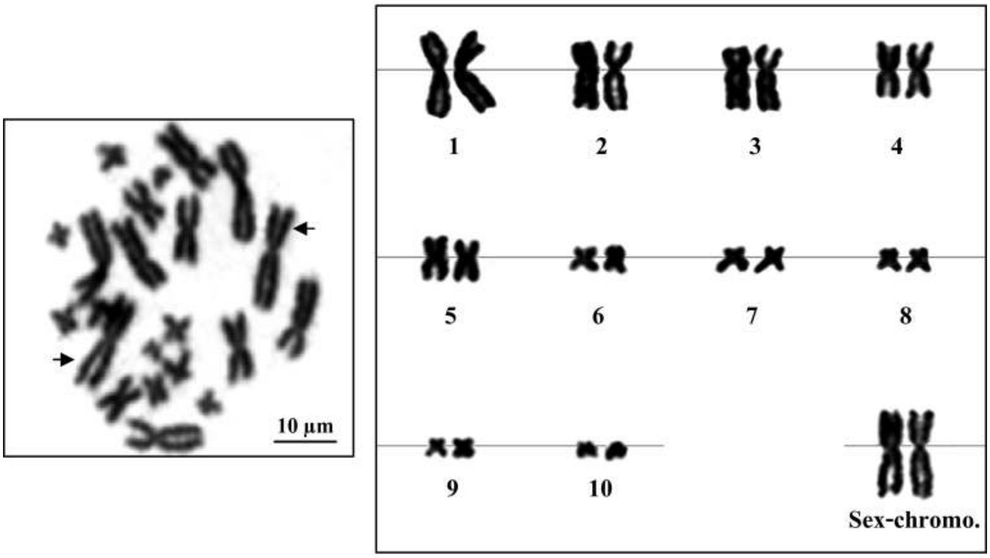
Fig. 4. Metaphase chromosome plates and karyotypes of male (A) and female (B) common Indian toad (*Bufo melanostictus* Schneider 1799) 2*n* (diploid)=22 by C-banding technique, showing sex-chromosomes (arrows).

Results and discussion

We found that all of four toad species in Thailand: *B. macrotis*, *B. parvus*, *B. melanostictus* and *B. asper*'s chromosome numbers are 2*n*=22 (Fig. 8). The result is consistent to the report of Bogat (1966), Beckert and Doyle (1967), Cole *et al.* (1968), Schmid (1978a), Supaporm and Kanlayaprasith (1990), Narkkasem (1975), which revealed that most species of genus *Bufo* have 2*n*=22 except



A. The male large-eared toad (*Bufo macrotis*)

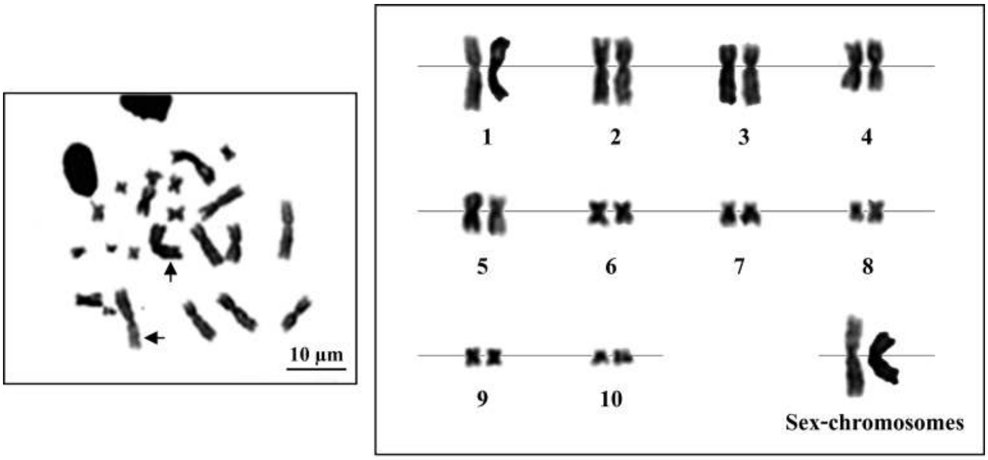


B. The female large-eared toad (*Bufo macrotis*)

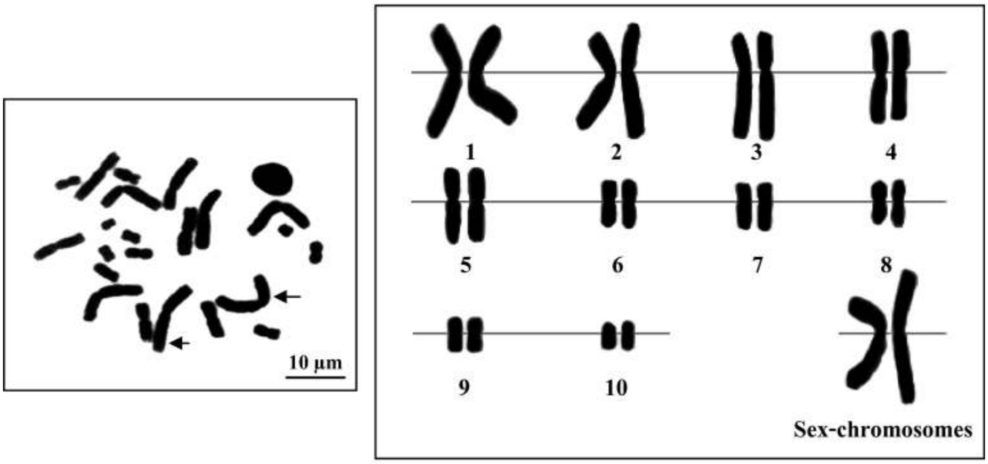
Fig. 5. Metaphase chromosome plates and karyotypes of male (A) and female (B) large-eared toad (*Bufo macrotis* Boulenger 1887) $2n$ (diploid)=22 by conventional staining technique, showing sex-chromosomes (arrows).

in 6 species which are existence in Africa: *B. regularis*, *B. gutturalis*, *B. garmani*, *B. rangeri*, *B. brauni* and *B. latifrons* that have $2n=20$. Several scientist guest that Africa is the original native of genus *Bufo* ($2n=20$). After that, they have change in chromosome number ($2n=22$) and then have been distributing throughout the world (Bogart 1966).

We also found that both of male and female of all of four toad species in Thailand: *B. macrotis*, *B. parvus*, *B. melanostictus* and *B. asper*'s fundamental number (NF) are 44. The autosome types of *B. macrotis* and *B. melanostictus* were being as 18 metacentric and 4 submetacentric



A. The male Indochinese dwarf toad (*Bufo parvus*)

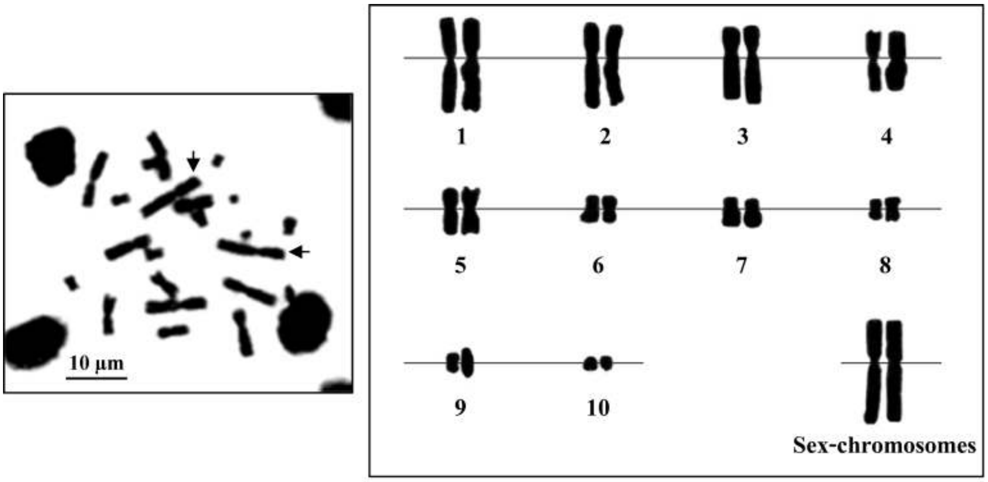


B. The female Indochinese dwarf toad (*Bufo parvus*)

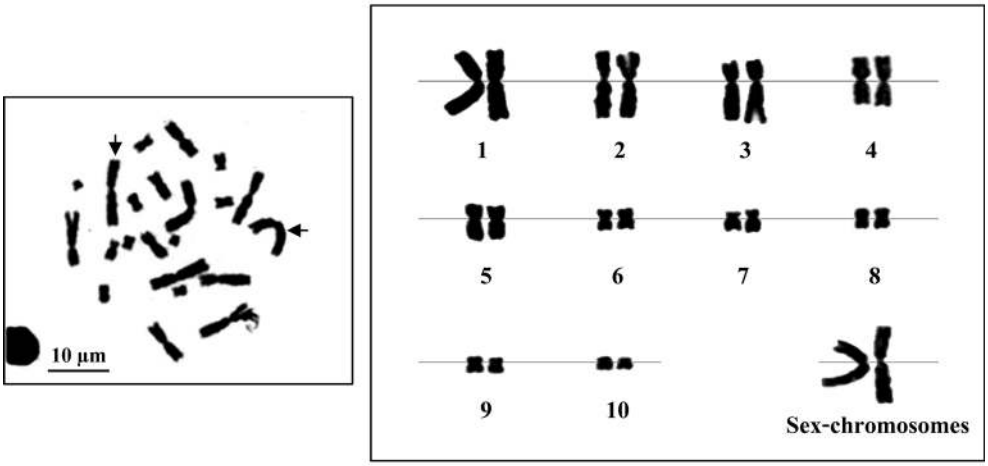
Fig. 6. Metaphase chromosome plates and karyotypes of male (A) and female (B) Indochinese dwarf toad (*Bufo parvus* Boulenger 1887) $2n$ (diploid)=22 by conventional staining technique, showing sex-chromosomes (arrows).

chromosomes (Figs. 2–5) while *B. parvus* and *B. asper* were as 16 metacentric and 6 submetacentric chromosomes (Figs. 6, 7). It is not much differ from the reports of Narkkasem (1975) and Supaporm and Kanlayaprasith (1990) that revealed the 18 metacentric and 4 submetacentric chromosomes of *B. melanostictus*'s autosome types. Moreover, it is quite consistent to Cole *et al.* (1968) that report the submetacentric and metacentric chromosomes of eight species of genus *Bufo* which are existence in North America.

The autosome size of *B. macrotis*, *B. parvus* and *B. melanostictus* are being as 5 large chromosome pairs, 1 medium pair and 5 small pairs (Tables 1–3) while *B. asper* is as 5 large chromosome pairs and 6 small pairs (Table 4). Our result have much consistent to Narkkasem (1975) and Supaporm and Kanlayaprasith (1990) that revealed the 5 large chromosome pairs, 1 medium pair and 5



A. The male river giant toad (*Bufo asper*)



B. The female river giant toad (*Bufo asper*)

Fig. 7. Metaphase chromosome plates and karyotypes of male (A) and female (B) river giant toad (*Bufo asper* Gravenhorst 1829) $2n$ (diploid)$=22$ by conventional staining technique, showing sex-chromosomes (arrows).

small pairs of *B. melanostictus*'s autosome sizes. At this point of our knowledge, the cytogenetic study of *B. macrotis* and *B. parvus*, this is the first report.

G-banding technique showed the *B. melanostictus*'s constriction on short arm of Y chromosome (the largest chromosome) but not show on X chromosome. Those feature is appeared in all male metaphase plate cells. So that, we agree that the sex determination of *B. melanostictus* is XY system (Figs. 3, 9). It is much similar to the reports of Iturra and Veloso (1989), Schmid *et al.* (1989, 1990, 1991, 1993) which demonstrated that several amphibian species has the same chromosome type and size but has a different band patterns by banding techniques. G-banding technique can show the labels of histone protein stained (only histone protein which exist on nitrogenous base AT-rich DNA regions) by Giemsa's dye (Summer 1990).

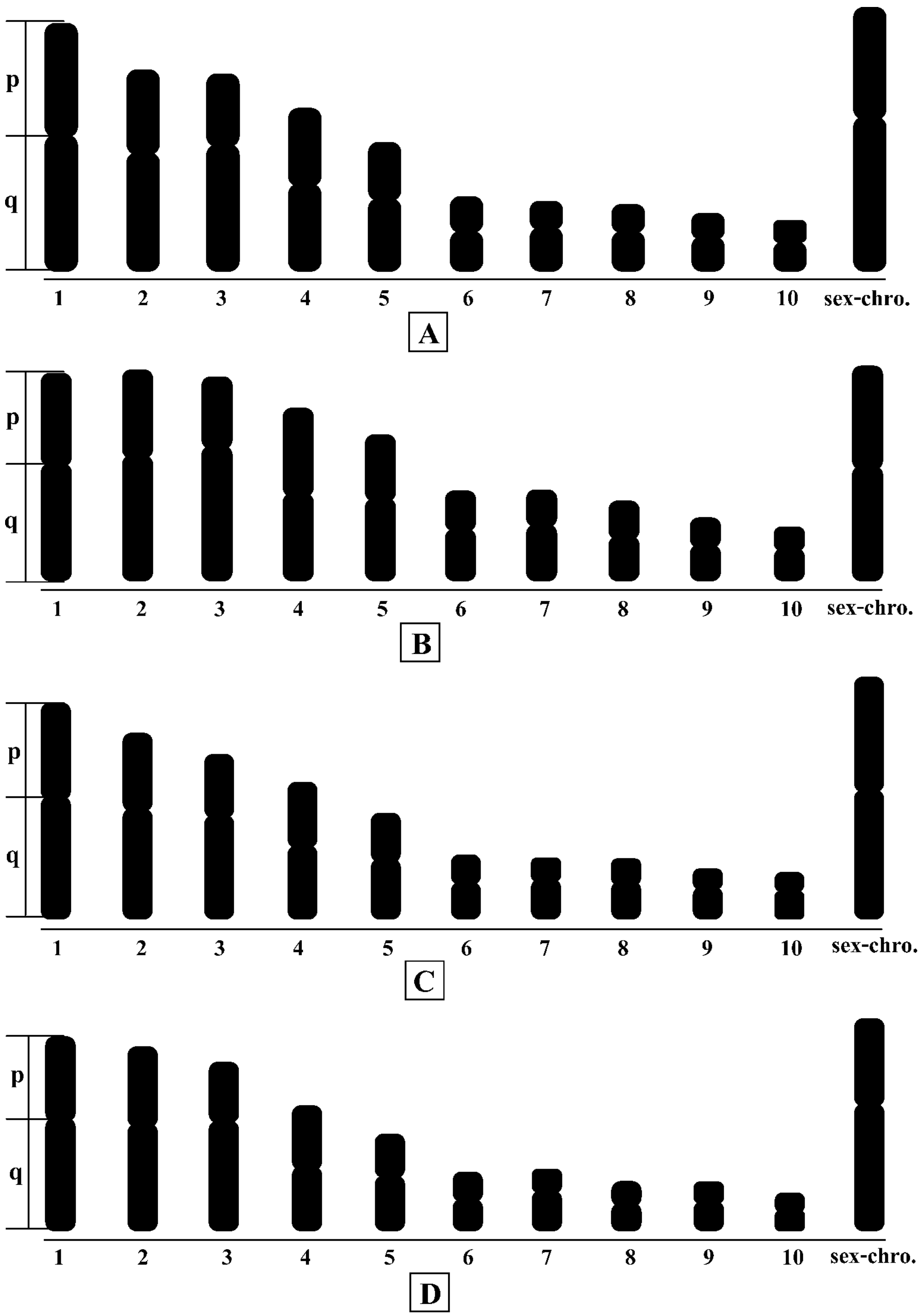


Fig. 8. Idiogram of four toad species of the genus *Bufo* in Thailand namely; large-eared toad, *Bufo macrotis* (A) Indochinese dwarf toad, *Bufo parvus* (B) common Indian toad, *Bufo melanostictus* (C) and river giant toad, *Bufo asper* (D). * sex-chro.=sex-chromosomes.

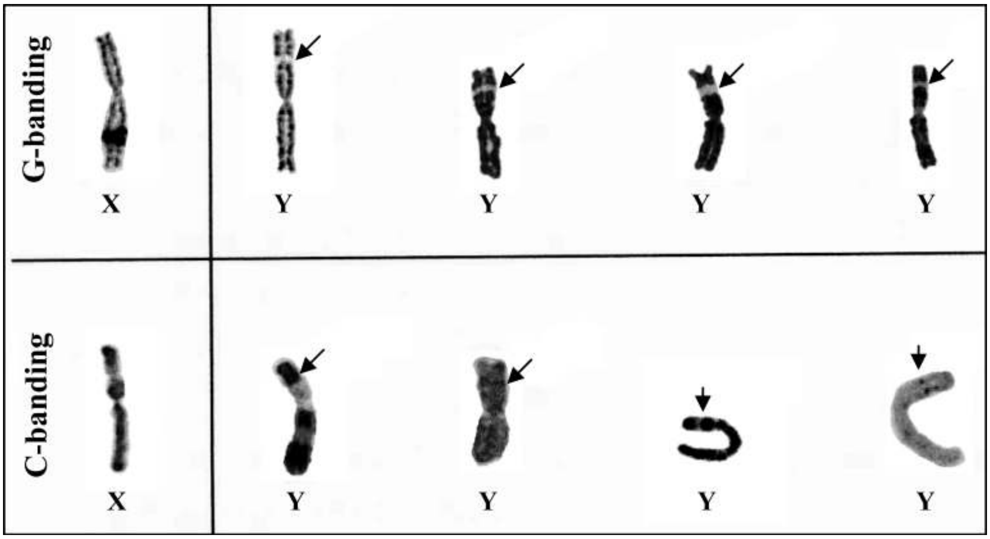


Fig. 9. Sex-chromosomes of common Indian toad (*Bufo melanostictus* Schneider 1799) indicated the presence of heteromorphism of X and Y chromosomes in male, there is a constriction and dark band (arrows) on Y chromosome by G-banding and C-banding techniques, respectively but not show on X chromosome.

Table 1. Mean of length short arm chromosome (Ls), length long arm chromosome (LL), length total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from metaphase chromosomes of 20 cells in male and female common Indian toad (*Bufo melanostictus*) $2n$ (diploid)=22

Chromosome pairs	Ls	LL	LT	RL+SD	CI+SD	Chromosome size	Chromosome type
1	0.69	0.89	1.56	0.088±0.012	0.557±0.052	large	metacentric
2	0.59	0.87	1.35	0.076±0.008	0.562±0.011	large	metacentric
3	0.45	0.76	1.20	0.067±0.005	0.625±0.176	large	submetacentric
4	0.51	0.75	1.05	0.059±0.003	0.514±0.182	large	metacentric
5	0.37	0.54	0.82	0.046±0.001	0.548±0.049	medium	metacentric
6	0.25	0.27	0.52	0.029±0.006	0.519±0.172	small	metacentric
7	0.09	0.39	0.48	0.027±0.007	0.812±0.156	small	metacentric
8	0.23	0.23	0.46	0.026±0.007	0.500±0.163	small	metacentric
9	0.18	0.23	0.41	0.023±0.008	0.560±0.020	small	metacentric
10	0.11	0.20	0.31	0.017±0.010	0.645±0.020	small	submetacentric
Sex-chromo.	0.76	0.89	1.65	0.093±0.014	0.539±0.106	large	metacentric

C-banding technique demonstrated a dark band constriction on Y chromosome of *B. melanostictus*, the representative of constitutive heterochromatin. However, there is no dark band constriction on X chromosome (Figs. 4, 9). The dark bands those appear by C-banding technique are obviously arises on centromeres, telomeres and some parts of its regions.

G-banding and C-banding techniques led us to conclude that the largest chromosome pair of male *B. melanostictus* is polymorphism (heteromorphism X and Y chromosome). The conclusion is consistent to Mahony (1991) that demonstrated sex-chromosome of *Crinia bilingual* using C-banding and NOR-banding techniques. According to his study, he concluded that the sex determination of those species is ZW system. His C-banding results showed a dark band on constitutive hete-

Table 2. Mean of length short arm chromosome (Ls), length long arm chromosome (LL), length total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from metaphase chromosomes of 20 cells in male and female large-eared toad (*Bufo macrotis*) 2*n* (diploid)=22

Chromosome pairs	Ls	LL	LT	RL+SD	CI+SD	Chromosome size	Chromosome type
1	0.70	0.80	1.50	0.084±0.010	0.533±0.090	large	metacentric
2	0.56	0.77	1.33	0.074±0.007	0.578±0.065	large	metacentric
3	0.47	0.75	1.22	0.068±0.005	0.614±0.198	large	submetacentric
4	0.52	0.55	1.07	0.060±0.003	0.514±0.147	large	metacentric
5	0.39	0.47	0.86	0.048±0.006	0.546±0.096	medium	metacentric
6	0.25	0.27	0.52	0.029±0.006	0.519±0.156	small	metacentric
7	0.18	0.32	0.50	0.028±0.006	0.640±0.129	small	submetacentric
8	0.20	0.24	0.44	0.024±0.008	0.545±0.084	small	metacentric
9	0.16	0.22	0.38	0.021±0.009	0.578±0.030	small	metacentric
10	0.13	0.18	0.31	0.017±0.010	0.580±0.150	small	metacentric
Sex-chromo.	0.79	0.91	1.70	0.095±0.010	0.530±0.071	large	metacentric

Table 3. Mean of length short arm chromosome (Ls), length long arm chromosome (LL), length total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from metaphase chromosomes of 20 cells in male and female Indochinese dwarf toad (*Bufo parvus*) 2*n* (diploid)=22

Chromosome pairs	Ls	LL	LT	RL+SD	CI+SD	Chromosome size	Chromosome type
1	0.72	0.92	1.64	0.087±0.012	0.560±0.055	large	metacentric
2	0.62	0.83	1.45	0.077±0.008	0.572±0.008	large	metacentric
3	0.49	0.85	1.34	0.071±0.006	0.634±0.005	large	submetacentric
4	0.57	0.60	1.17	0.062±0.003	0.512±0.116	large	metacentric
5	0.40	0.49	0.89	0.047±0.009	0.550±0.176	medium	metacentric
6	0.26	0.30	0.56	0.030±0.006	0.535±0.071	small	metacentric
7	0.17	0.32	0.49	0.026±0.007	0.653±0.134	small	submetacentric
8	0.21	0.24	0.45	0.024±0.008	0.533±0.143	small	metacentric
9	0.17	0.19	0.36	0.019±0.009	0.527±0.071	small	metacentric
10	0.11	0.17	0.28	0.015±0.011	0.607±0.049	small	submetacentric
Sex-chromo.	0.79	0.96	1.75	0.093±0.014	0.548±0.111	large	metacentric

Table 4. Mean of length short arm chromosome (Ls), length long arm chromosome (LL), length total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from metaphase chromosomes of 20 cells in male and female river giant toad (*Bufo asper*) 2*n* (diploid)=22

Chromosome pairs	Ls	LL	LT	RL+SD	CI+SD	Chromosome size	Chromosome type
1	0.73	0.95	1.68	0.087±0.012	0.565±0.091	large	metacentric
2	0.60	0.87	1.47	0.076±0.008	0.591±0.072	large	metacentric
3	0.49	0.85	1.34	0.069±0.006	0.634±0.014	large	submetacentric
4	0.56	0.56	1.12	0.058±0.002	0.500±0.184	large	metacentric
5	0.37	0.50	0.87	0.045±0.001	0.574±0.139	medium	metacentric
6	0.23	0.33	0.56	0.029±0.006	0.589±0.043	small	metacentric
7	0.16	0.35	0.51	0.026±0.007	0.686±0.009	small	submetacentric
8	0.23	0.23	0.46	0.024±0.008	0.500±0.142	small	metacentric
9	0.18	0.23	0.41	0.021±0.009	0.560±0.101	small	metacentric
10	0.11	0.20	0.31	0.016±0.011	0.645±0.088	small	submetacentric
Sex-chromo.	0.85	1.07	1.92	0.099±0.015	0.557±0.100	large	metacentric

rochromatin region of short arm and W chromosome but not show on Z chromosome. Moreover, he showed that a dark band by C-banding will become a constriction when stained by NOR-banding technique.

Our results indicated that *B. melanostictus*'s chromosome is being in the evolutionary change. Moreover, its X and Y chromosome have the same type and also equal size but have different band patterns by banding techniques. For *B. macrotis*, *B. parvus* and *B. asper*, we can not study them by G-banding and C-banding techniques because of the few amount of samples and unsuccessful operation. However, we have an agreement that the sex-determination of *B. macrotis*, *B. parvus* and *B. asper* should be XY system.

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