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Cytogenetic Studies of Wrinkle-lipped Bat, *Chaerephon plicatus*, from Tham Pra Thun Non-hunting Area, Uthai Thani Province, Thailand

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ABSTRACT

Chromosome and morphometric analyses of the wrinkle-lipped bat, *Chaerephon plicatus* (Buchannan, 1800), from Khao Khong Chai Cave, Tham Pra Thun Non-hunting Area in Uthai Thani Province, Thailand were conducted. Bat chromosomes in both males and females were determined by the squash technique using bone marrow and testis following conventional staining and Ag-NOR banding. The results showed that the diploid chromosome number of *C. plicatus* was $2n = 48$, and the autosomal fundamental number (NFa) was 56 in both males and females. The types of autosomes were 4 large metacentric, 2 medium metacentric, 8 medium telocentric, 4 small metacentric, and 28 small telocentric chromosomes. The X chromosome was medium metacentric, and the Y chromosome was small telocentric. The nucleolar organizer region (NOR) chromosome markers were found on chromosome pair 6 telocentric and 9 metacentric. The karyotype of *C. plicatus* was as follows: $2n$ (48) = $L_4^m + M_2^m + M_8^t + S_4^m + S_{28}^t +$ sex chromosomes. These findings provide additional cytogenetic information for *C. plicatus* and contribute to the understanding of chromosomal variation within the family Molossidae.

Keywords: *Chaerephon plicatus*; Karyotype; Ideogram; Nucleolar organizer region; Wrinkle-lipped bat

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1. Introduction

Bats are the only mammals capable of flying and communicating by echolocation. They are classified in the order Chiroptera (bats), which consists of eighteen recognized families of bats, and more than 1,116 species that account for 21% of identified mammal species [1]. Numerous reports in morphological, biochemical, cytogenetics, and molecular studies have provided support for the order Chiroptera, which has the unique trait of powered flight in mammals. The order Chiroptera was established no less than 50-60 million years ago. The power of flight enabled bats to fill an extremely fertile niche and led to a great proliferation of forms. Thailand has a very rich diversity of bat fauna, including 2 suborders, 10 families, and 33 genera. The two suborders include Megachiroptera (fruit bats) and Microchiroptera (insect-eating bats) [2]. The wrinkle-lipped bat is classically described as belonging to the suborder Microchiroptera, family Molossidae (free-tailed bats), described by Buchannan [3] as *Vespertilio plicatus*. Subsequently, this species was reclassified as *Tadarida plicata*, *Chaerephon plicata*, and *Chaerephon plicatus* [4]. Freeman [5] delineated that *Tadarida* and *Chaerephon* are two distinct genera. The colony size of *C. plicatus* ranges from 200,000 to over three million individuals. In Thailand, *C. plicatus* is found throughout of the country, but is most common in the central region [2].

Khao Khong Chai Cave is located within the Tham Pra Thun Non-hunting area, Uthai Thani Province, Thailand. Khao Khong Chai Cave is approximately 353 meters high. The front side of Khao Khong Chai features a wide, shallow cave resembling a large tunnel. It is believed that prehistoric humans once inhabited the area, as stone tools and pottery fragments have been discovered there. Inside the cave, a large number of bats reside. In the evening, flocks of bats can be seen flying out in search of food for hours. This study is the first report on the determination of chromosome size, karyotype formula, and standardized ideogram of *C. plicatus* from this area. The results represent significant cytogenetic information that will be useful in further study on taxonomy, conservation, and studying chromosomal evolution in the order Chiroptera.

2. Materials and Methods

2.1 Animal sample collection

Three males and three females of wrinkle-lipped bat (*C. plicatus*) were captured using bird nets at Khao Khong Chai Cave, located in Tham Pra Thun Non-hunting Area, Uthai Thani Province. The capture was conducted with permission from the Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Thailand, under Permission Letter No. ทส 0907.404/26231. This research protocol was approved by the Nakhon Sawan Rajabhat University Animal Care and Use Committee under approval numbers NSRU-IACUC No. 202115 for bone marrow samplings and NSRU-IACUC No. 202501 for testis samplings. Species identification was done following methodology from Lekagul and McNeely [2].

2.2 Chromosome preparations

All bats were injected with 0.1 ml per 10 g of body weight of 0.1% colchicine and maintained at room temperature for 12 hours. Subsequently, euthanasia was performed using carbon dioxide. Bone marrow samples from both male and female bats were collected from the humerus, forearm, metacarpal, and femur for chromosome preparation using the squash technique. In addition, testis samples were also collected for chromosome analysis. The metaphase cells were collected and kept in Carnoy's fixative at -20°C.

2.3 Chromosome staining

The cell suspension was dropped onto a slide pre-treated with Carnoy's fixative and dried on a hotplate until fully evaporated. The cells were stained using the conventional method with 20% Giemsa's solution for 15-30 min and silver-nucleolar organizer region (Ag-NOR) staining with 50% silver nitrate ($AgNO_3$) to investigate the chromosome marker [6, 7]. The chromosomes were examined under a microscope using 100X objective lens.

2.4 Chromosome analysis

Twenty metaphase cells from the bone marrow of each sex and fifteen metaphase cells from the testis were used for chromosome analysis. The morphometric analysis of chromosomes was measured and analyzed by the length of the short arm (Ls), long arm (Ll), length of the total arm (LT = Ls + Ll), relative length (RL), and centromeric index (CI). The CI was computed to classify the types of chromosomes according to Turpin & Lejeune [8]. All parameters were used for karyotype and ideogram analysis.

3. Results and Discussion

3.1 Morphological description of *C. plicatus*

C. plicatus belongs to the family Molossidae, which includes two genera and two species in Thailand: *Cheiromeles torquatus* and *Tadarida plicata*. However, *T. plicata* has been reclassified as *C. plicatus* [4]. A distinctive characteristic of the family Molossidae is their free-tailed morphology (Fig. 1A). *C. plicatus* is particularly notable for its large lips, with the upper lip deeply furrowed by vertical wrinkles, giving rise to its common name, the wrinkle-lipped bat (Figs. 1B and 1C). The average head-body (HB) length of *C. plicatus* ranges from 65 to 75 mm, with a tail (T) length of 30 to 40 mm and a forearm (FA) length of 40 to 50 mm. The ear (E) length measures between 21 and 24 mm, while its weight (W) ranges from 17 to 31 g [2]. In this study, specimens collected from Khao Khong Chai Cave had an average HB length of 67.99 ± 0.79 mm, a left FA length of 46.33 ± 2.36 mm, a right FA length of 45.66 ± 2.27 mm, and a T length of 34.65 ± 3.17 mm. These measurements fall within the reported reference ranges for *C. plicatus* species.

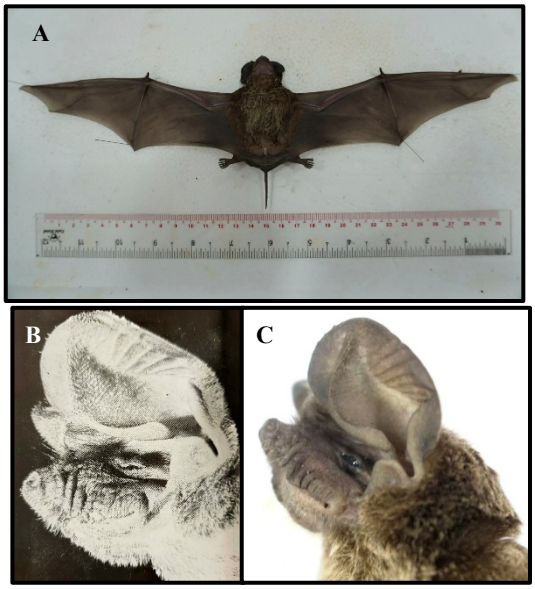


Fig. 1. Morphological characteristics of the family Molossidae: free-tailed morphology (A); the distinctive upper lip of *C. plicatus*, deeply furrowed with vertical wrinkles, as described by Lekagul and McNeely [2] (B); and *C. plicatus* from Khao Khong Chai cave (C).

3.2 Karyotype and ideogram of *C. plicatus*

The cytogenetic study of *C. plicatus* using the squash technique from bone marrow and the conventional chromosome staining (20% Giemsa) revealed that the diploid (*2n*) chromosome number was 48 in both males and females. The diploid chromosome comprised 46 (23 pairs) autosomes and 2 (1 pair) sex chromosomes. The autosomal fundamental number (NFa) of *C. plicatus* was 56 in both males (Fig. 2A) and females (Fig. 2B). Similarly, the cytogenetic study of *C. plicatus* using the squash technique from testis samples also showed the same results

of diploid (*2n*) chromosome number and the fundamental number (NF) as found in bone marrow samples (Fig. 3). The diploid (*2n*) chromosome number observed in this study was consistent with previous reports [9, 10]. However, the NF differed from those reported: one study reported an NF of 54 [9], and another reported an NFa of 54 [10].

In this study, the autosomes of *C. plicatus* from Khao Khong Chai Cave consisted of four large metacentric pairs, two medium metacentric pairs, eight medium telocentric pairs, four small metacentric pairs, and twenty-eight small telocentric pairs (Tables 1-2). In contrast, Harada et al. [9] reported that the autosomes of *T. plicata* (the former scientific name of *C. plicatus*) from Lop Buri Province consisted of four meta-submetacentric pairs and nineteen acrocentric pairs of medium to small size. Similarly, Sreepada et al. [10] described the autosomes of *C. plicata* from Kolar, India, as comprising one large metacentric pair, three medium sized meta-to submetacentric pairs, and nineteen medium to small sized acrocentric pairs.

The sex chromosomes of *C. plicatus* were characterized as follows: the X chromosome was identified as a medium-sized metacentric chromosome in both males and females, whereas the Y chromosome was small telocentric (Tables 1, 2, and 3). In contrast, Harada et al. [9] described the X chromosome as medium-sized submetacentric and the Y chromosome as small acrocentric. Similarly, Sreepada et al. [10] reported the X chromosome as a medium-sized metacentric as found in this study, and the Y chromosome as a small acrocentric. The standard ideograms obtained from conventionally stained bone marrow preparations of male and female *C. plicatus* are presented in Figs. 3A-3B, respectively, while the standard ideogram derived from the testicular cells of the male is shown in Fig. 4.

Comparison of the present results with previous reports revealed discrepancies in chromosome classification. The differences in chromosome classification among studies likely resulted from the use of different CI reference systems for determining chromosome types. In the present study, chromosomes were classified following the criteria of Turpin and Lejeune [8], whereas Harada et al. [9] adopted the classification system proposed by Levan et al. [11]. In contrast, Sreepada et al. [10] did not specify the reference used for determining CI. The main difference between the chromosome classification systems of Turpin and Lejeune [8] and Levan et al. [11] lies in the classification of chromosomes with terminal or subterminal centromeres. For example, chromosomes classified as acrocentric under the Turpin and Lejeune system may be classified as subtelocentric under the Levan et al. system. These differences may contribute to discrepancies in the reported karyotype formulas and NF/NFa values due to differences in the classification of autosomes and sex chromosomes. The NF/NFa is an important cytogenetic parameter because it reflects the total number of chromosome arms and provides additional information on karyotype structure beyond the diploid (*2n*) chromosome number. Therefore, the differences in NF/NFa may indicate variations in chromosome morphology, chromosome classification, or chromosomal rearrangements, even when the diploid (*2n*) chromosome number remains unchanged.

Additionally, previous cytogenetic studies on three species of bat in family Molossidae; *Mormopterus planiceps*, *Mormopterus jugularis*, and *Mops Micropterus*, have reported a consistent diploid chromosome number of 48 ($2n = 48$) [12], which is in accordance with the findings of the present study. In that report, only the karyotype of *M. planiceps* was described in detail, comprising four metacentric and 19 acrocentric autosomal pairs, whereas the karyotypes of the other two species were not provided [12]. Taken together, these

observations suggest that bats within the family Molossidae generally possess a diploid (*2n*) chromosome number of 48, although their NF and karyotypic characteristics may vary among species.

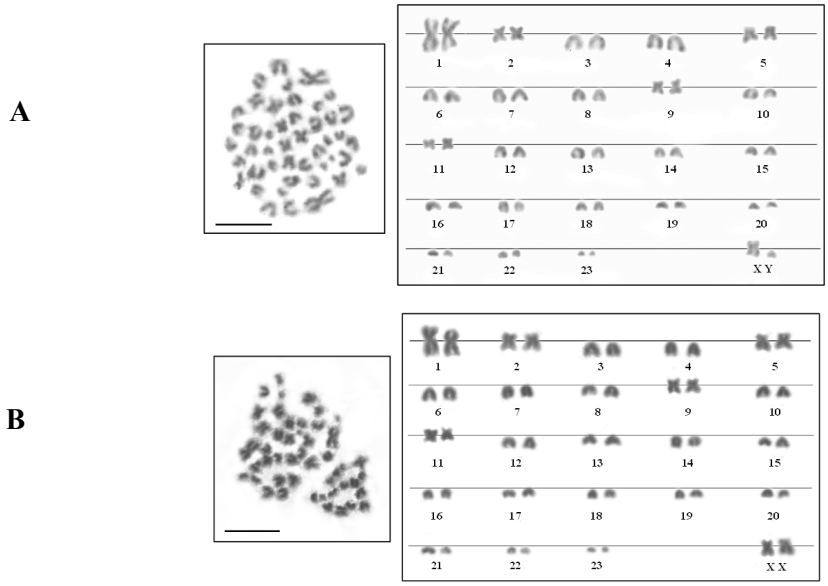


Fig. 2. Metaphase chromosome plates and karyotypes from bone marrow of male (A) and female (B) wrinkle-lipped bat (*C. plicatus*), *2n*=48 by conventional staining technique (scale bar 10 μm).

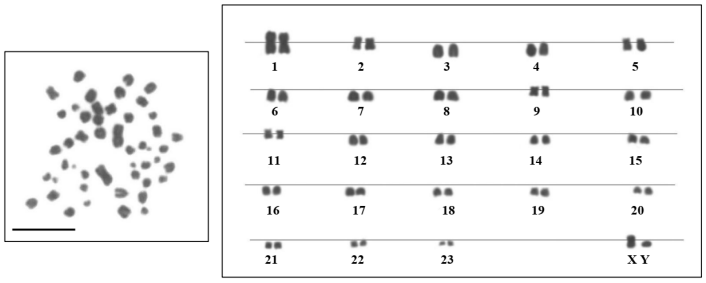


Fig. 3. Metaphase chromosome plates and karyotypes from testis of male wrinkle-lipped bat (*C. plicatus*), *2n*=48 by conventional staining technique (scale bar 10 μm).

Table 1. Morphometric analysis of metaphase chromosomes from 20 bone marrow cells of male wrinkle-lipped bat (*C. plicatus*), with *2n* (diploid) = 48. Chromosome measurements are presented in micrometers (μm).

Chromosome pair	Ls	Ll	LT	RL±SD	CI±SD	Chromosome size	Chromosome type
1	1.109	1.393	2.502	0.099 ± 0.005	0.557 ± 0.014	Large	Metacentric
2	0.629	0.783	1.412	0.056 ± 0.009	0.555 ± 0.017	Large	Metacentric
3	0.000	1.371	1.371	0.054 ± 0.004	1.000 ± 0.000	Medium	Telocentric
4	0.000	1.345	1.345	0.053 ± 0.003	1.000 ± 0.000	Medium	Telocentric
5	0.600	0.691	1.291	0.051 ± 0.003	0.535 ± 0.016	Medium	Metacentric
6	0.000	1.291	1.291	0.051 ± 0.003	1.000 ± 0.000	Medium	Telocentric
7	0.000	1.265	1.265	0.050 ± 0.002	1.000 ± 0.000	Medium	Telocentric
8	0.000	1.127	1.127	0.045 ± 0.003	1.000 ± 0.000	Small	Telocentric
9	0.484	0.616	1.100	0.044 ± 0.003	0.560 ± 0.020	Small	Metacentric

10	0.000	1.111	1.111	0.044 ± 0.004	1.000 ± 0.000	Small	Telocentric
11	0.440	0.564	1.004	0.040 ± 0.003	0.561 ± 0.015	Small	Metacentric
12	0.000	0.997	0.997	0.040 ± 0.004	1.000 ± 0.000	Small	Telocentric
13	0.000	0.981	0.981	0.039 ± 0.003	1.000 ± 0.000	Small	Telocentric
14	0.000	0.950	0.950	0.038 ± 0.004	1.000 ± 0.000	Small	Telocentric
15	0.000	0.879	0.879	0.035 ± 0.004	1.000 ± 0.000	Small	Telocentric
16	0.000	0.846	0.846	0.034 ± 0.003	1.000 ± 0.000	Small	Telocentric
17	0.000	0.801	0.801	0.032 ± 0.003	1.000 ± 0.000	Small	Telocentric
18	0.000	0.776	0.776	0.031 ± 0.003	1.000 ± 0.000	Small	Telocentric
19	0.000	0.599	0.599	0.024 ± 0.002	1.000 ± 0.000	Small	Telocentric
20	0.000	0.520	0.520	0.021 ± 0.003	1.000 ± 0.000	Small	Telocentric
21	0.000	0.475	0.475	0.019 ± 0.002	1.000 ± 0.000	Small	Telocentric
22	0.000	0.385	0.385	0.015 ± 0.002	1.000 ± 0.000	Small	Telocentric
23	0.000	0.252	0.252	0.010 ± 0.003	1.000 ± 0.000	Small	Telocentric
X	0.647	0.660	1.307	0.052 ± 0.003	0.505 ± 0.025	Medium	Metacentric
Y	0.000	0.617	0.617	0.024 ± 0.004	1.000 ± 0.000	Small	Telocentric

Table 2. Morphometric analysis of metaphase chromosomes from 20 bone marrow cells of female wrinkle-lipped bat (*C. plicatus*), with *2n* (diploid) = 48. Chromosome measurements are presented in micrometers (μm).

Chromosome pair	Ls	Ll	LT	RL±SD	CI±SD	Chromosome size	Chromosome type
1	1.082	1.196	2.278	0.099 ± 0.004	0.525 ± 0.015	Large	Metacentric
2	0.705	0.865	1.570	0.068 ± 0.011	0.551 ± 0.017	Large	Metacentric
3	0.000	1.242	1.242	0.054 ± 0.003	1.000 ± 0.000	Medium	Telocentric
4	0.000	1.216	1.216	0.053 ± 0.002	1.000 ± 0.000	Medium	Telocentric
5	0.540	0.674	1.215	0.053 ± 0.002	0.555 ± 0.019	Medium	Metacentric
6	0.000	1.183	1.183	0.052 ± 0.001	1.000 ± 0.000	Medium	Telocentric
7	0.000	1.166	1.166	0.051 ± 0.002	1.000 ± 0.000	Medium	Telocentric
8	0.000	1.043	1.043	0.045 ± 0.002	1.000 ± 0.000	Small	Telocentric
9	0.452	0.553	1.005	0.044 ± 0.005	0.550 ± 0.016	Small	Metacentric
10	0.000	0.934	0.934	0.041 ± 0.004	1.000 ± 0.000	Small	Telocentric
11	0.412	0.515	0.928	0.040 ± 0.003	0.555 ± 0.019	Small	Metacentric
12	0.000	0.805	0.805	0.035 ± 0.004	1.000 ± 0.000	Small	Telocentric
13	0.000	0.765	0.765	0.033 ± 0.004	1.000 ± 0.000	Small	Telocentric
14	0.000	0.704	0.704	0.031 ± 0.003	1.000 ± 0.000	Small	Telocentric
15	0.000	0.656	0.656	0.029 ± 0.005	1.000 ± 0.000	Small	Telocentric
16	0.000	0.585	0.585	0.025 ± 0.004	1.000 ± 0.000	Small	Telocentric
17	0.000	0.563	0.563	0.025 ± 0.003	1.000 ± 0.000	Small	Telocentric
18	0.000	0.534	0.534	0.023 ± 0.002	1.000 ± 0.000	Small	Telocentric
19	0.000	0.495	0.495	0.022 ± 0.003	1.000 ± 0.000	Small	Telocentric
20	0.000	0.476	0.476	0.021 ± 0.003	1.000 ± 0.000	Small	Telocentric
21	0.000	0.434	0.434	0.019 ± 0.002	1.000 ± 0.000	Small	Telocentric
22	0.000	0.389	0.389	0.017 ± 0.002	1.000 ± 0.000	Small	Telocentric
23	0.000	0.348	0.348	0.015 ± 0.002	1.000 ± 0.000	Small	Telocentric
X	0.558	0.657	1.215	0.053 ± 0.003	0.541 ± 0.016	Medium	Metacentric
X	0.549	0.651	1.200	0.052 ± 0.002	0.542 ± 0.017	Medium	Metacentric

Table 3. Morphometric analysis of metaphase chromosomes from 15 testicular cells of male wrinkle-lipped bat (*C. plicatus*), with *2n* (diploid) = 48. Chromosome measurements are presented in micrometers (μm).

Chromosome pair	Ls	Ll	LT	RL±SD	CI±SD	Chromosome size	Chromosome type
1	0.866	0.926	1.792	0.099±0.007	0.517±0.013	Large	Metacentric
2	0.598	0.678	1.276	0.070±0.005	0.513±0.016	Large	Metacentric
3	0.000	1.027	1.027	0.057±0.004	1.000±0.000	Medium	Telocentric
4	0.000	0.996	0.996	0.055±0.003	1.000±0.000	Medium	Telocentric
5	0.466	0.506	0.972	0.053±0.003	0.521±0.010	Medium	Metacentric
6	0.000	0.945	0.945	0.052±0.003	1.000±0.000	Medium	Telocentric
7	0.000	0.915	0.915	0.050±0.003	1.000±0.000	Medium	Telocentric
8	0.000	0.851	0.851	0.047±0.003	1.000±0.000	Small	Telocentric

9	0.400	0.433	0.834	0.046±0.004	0.520±0.018	Small	Metacentric
10	0.000	0.791	0.791	0.044±0.002	1.000±0.000	Small	Telocentric
11	0.357	0.404	0.761	0.042±0.002	0.531±0.019	Small	Metacentric
12	0.000	0.677	0.677	0.037±0.003	1.000±0.000	Small	Telocentric
13	0.000	0.628	0.628	0.035±0.003	1.000±0.000	Small	Telocentric
14	0.000	0.589	0.589	0.032±0.003	1.000±0.000	Small	Telocentric
15	0.000	0.553	0.553	0.030±0.004	1.000±0.000	Small	Telocentric
16	0.000	0.515	0.515	0.028±0.003	1.000±0.000	Small	Telocentric
17	0.000	0.485	0.485	0.027±0.003	1.000±0.000	Small	Telocentric
18	0.000	0.452	0.452	0.025±0.003	1.000±0.000	Small	Telocentric
19	0.000	0.427	0.427	0.023±0.003	1.000±0.000	Small	Telocentric
20	0.000	0.398	0.398	0.022±0.003	1.000±0.000	Small	Telocentric
21	0.000	0.371	0.371	0.020±0.003	1.000±0.000	Small	Telocentric
22	0.000	0.333	0.333	0.018±0.003	1.000±0.000	Small	Telocentric
23	0.000	0.288	0.288	0.016±0.003	1.000±0.000	Small	Telocentric
X	0.448	0.492	0.940	0.052±0.003	0.523±0.025	Medium	Metacentric
Y	0.000	0.356	0.356	0.020±0.004	1.000±0.000	Small	Telocentric

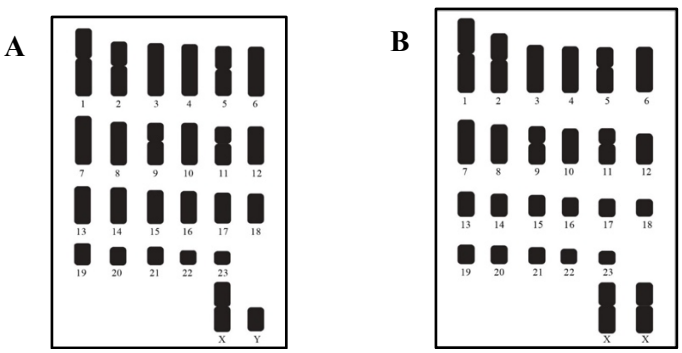


Fig. 4. Standard ideogram from bone marrow of male (A) and female (B) wrinkle-lipped bat (*C. plicatus*), *2n*=48 by conventional staining technique.

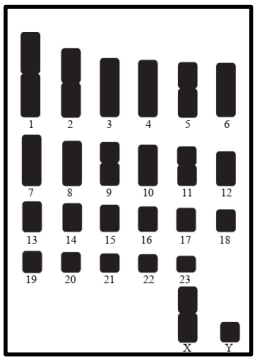


Fig. 5. Standard ideogram from testis of male wrinkle-lipped bat (*C. plicatus*), *2n*=48 by conventional staining technique.

3.3 Ag-NOR banded karyotype and ideogram of *C. plicatus*

The Ag-NOR staining technique was used to identify the nucleolar organizer region (NOR), indicating the location of genes involved in the synthesis of 18S and 28S ribosomal RNA. These chromosomal sites contain a higher proportion of non-histone proteins compared to other chromosomal regions. The major ribosomal DNA genes are clustered at these NORs. These proteins facilitate the formation of distinct dark bands (NOR-positive) through the reduction of organic silver, causing it to darken [13, 14, 15]. The standard ideograms obtained from Ag-NOR staining of both male and female

wrinkle-lipped bats (*C. plicatus*) are presented in Fig. 6 (A and B) from bone marrow preparations and in Fig. 7 from testicular tissue. The Ag-NOR bands were observed on the long arm (q) of the medium telocentric pair 6 and the short arm (p) of the small metacentric pair 9 as shown in Fig. 6 and 7. This study provides the first description of the NOR positions in this bat species. In Thailand, the chromosomal locations of NOR in several bat species have been characterized using the Ag-NOR banding technique. In *Scotophilus kuhlii*, NORs are located on the long arm of chromosome pair 17 [16]. In *Hipposideros larvatus*, NORs are located on the long arm of the large metacentric chromosome pair 8 [17]. For both *Rhinolophus coelophyllus* and *Rhinolophus pusillus*, NORs are located on the long arm of the large acrocentric chromosome pair 13 [18]. The Ag-NOR staining technique is widely used because it is rapid, reliable, simple, and inexpensive. Additionally, the benefits of this technique include (1) consistent and uniform silver staining of NOR, (2) few reduced silver deposits elsewhere on the chromosomes or on the slides, (3) generally unaltered chromosome morphology after silver treatment, and (4) relative permanence of Permounted preparation [13]. Moreover, the chromosomal distribution of NOR provides fundamental cytogenetic information for the species and represents a valuable marker for comparative cytogenetic studies, evaluation of karyotype stability and variation, and aids investigations of cytotaxonomic relationships and chromosome evolution among taxa.

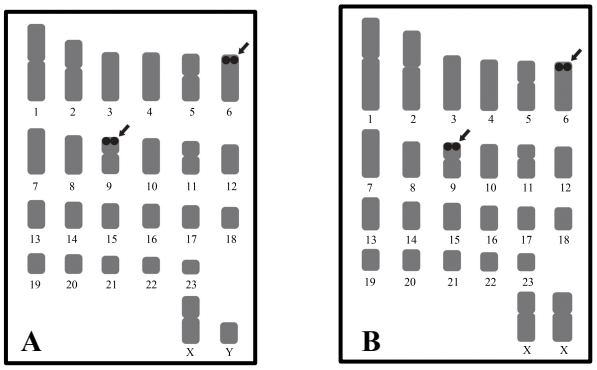


Fig. 6. Standard ideogram from bone marrow of male (A) and female (B) wrinkle-lipped bat (*C. plicatus*), *2n*=48 by Ag-NOR banding technique. Arrows indicate NOR.

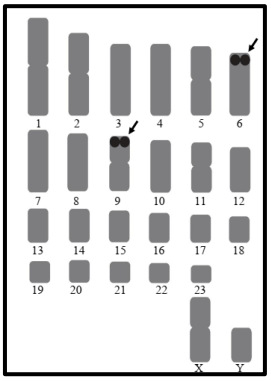


Fig. 7. Standard ideogram from testis of male wrinkled-lipped bat (*C. plicatus*), *2n*=48 by Ag-NOR banding technique. Arrows indicate NOR.

4. Conclusion

This study provides cytogenetic data for *C. plicatus* collected from Khao Khong Chai Cave in the Tham Pra Thun Non-hunting Area, Uthai Thani Province, Thailand. The species exhibits a diploid chromosome number of 48 ($2n$ = 48) and the autosomal fundamental number of 56 (NFa = 56) in both males and females. Notably, this work is the first to identify the NOR positions in *C. plicatus*. Two NORs were observed, located on the long arm of the medium telocentric chromosome pair 6 and the short arm of the small metacentric chromosome pair 9, providing useful chromosomal markers for this species.

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