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Chromosomal description and molecular cytogenetic markers of Nepalese whiskered myotis, *Myotis muricola* (Chiroptera: Vespertilionidae) from Thailand

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Abstract-The objectives of this study were to investigate size, shape, diploid number (2*n*), fundamental number (NF), NORs position and pattern of microsatellites and to establish the karyotype and standard ideogram of Nepalese whiskered myotis, *Myotis muricola* (Gray, 1846) from Maha Sarakham province, Northeastern Thailand. Mitotic chromosomes were directly prepared from bone marrow of specimens after *in vivo* colchicine treatment. Chromosomes were stained by conventional Giemsa and Ag-NOR staining as well as Fluorescence *in situ* hybridization (FISH) techniques. The obtained results showed that the diploid chromosome number of *M. muricola* was 2*n* = 44 and the fundamental number (NF) was 53 in male and 54 in female. The types of autosomes were 6 large metacentric, 2 small submetacentric and 34 small telocentric chromosomes. The sex determination was XY system which X chromosome was a large metacentric chromosome and Y was a small telocentric chromosome. NOR positions were located on the centromere of the long arm of chromosomes 8th and 16th. The hybridization signal of $d(CGG)_{10}$ was distributed across all chromosomes as well as the whole genome. In contrast, $d(GC)_{15}$ repeat was specifically presented on chromosomes 1st, 4th and 10th. The karyotype formula of *M. muricola* is as follows: 2*n* (44) = L^{m}_{6} + S^{sm}_{2} + S^{t}_{34} + sex chromosomes.

Keywords: Bat, cytogenetics, FISH, karyotype, microsatellite

1. Introduction

Vespertilionid bats are the most extensive species diversity within the order Chiroptera. This family is consisted of 48 genera and over 400 recognized species (Simmons, 2005). Among these, *Myotis* Kaup, 1829 is the most speciose genus of bats and the second largest mammal's genus, with more than 140 extant species (MDD, 2022; Novaes *et al.*, 2022) and 12 species occurring in Thailand (Soisook, 2011). It is the genus with the most incredible area of distribution among non-human mammals and has a wide distribution (Moratelli *et al.*, 2019). Its species exhibit significant similarities in forms and rarely display specialized features, hindering the process of identification and the systematic organization of the group (Faria & Ribeiro, 2018).

The Nepalese whiskered myotis, *Myotis muricola* (Gray, 1846) is a species of insectivorous bats in which Nepal is the type locality. The upper side of *M. muricola* is colored brown or grey with dark bases, while the bottom has dark bases and light brown tips. The ears are moderately long, slender, bent forwards and bluntly pointed (Francis, 2008). *M. muricola* has tiny feet with wing membranes attached at the base of the toes. The tail is long and completely enclosed in the interfemoral membrane. It also has three pairs of premolars, with the upper canine much longer than the third premolar. The second premolar is small and slightly intruded from the tooth row (Yasuma *et al.*, 2003). The most remarkable diversity and abundance ranged in Southeast Asia from East India to South China and Indonesia as well as from Sumatra through the Moluccas and the Philippines (Simmons, 2005; Francis, 2008). Despite being a broad and diverse genus, *Myotis* is one of the most conserved karyotypes (Baker & Jordan, 1970; Faria & Ribeiro, 2018). Phylogenetic studies classified the genus as being monophyletic in general (Stadelmann *et al.*, 2007; Faria & Ribeiro, 2018).

However, cytogenetic studies are still relatively scarce among *Myotis*, often using classical methodologies to determine the diploid number ($2n$) and karyotype composition. Studies applying molecular cytogenetic approaches have been done on only one species, *Myotis myotis* (Volleth *et al.*, 2002). Up to date, only 16 species from 140 species have been cytogenetically examined (Table 1). The overall data showed the stable $2n = 44$ (Harada *et al.*, 1985; Bickham, 1986; Wu *et al.*, 2006; 2009; Karataş *et al.*, 2013; Supanuam *et al.*, 2013; Baydemir, 2015; Albayrak *et al.*, 2020) except for *M. annectans* and *M. ricketti* which possessed $2n = 46$ (Bickham *et al.*, 1986; Wu *et al.*, 2006).

Here we add new data for some molecular chromosome markers analyzed by Fluorescence *in situ* hybridization (FISH) technique for the first time, as well as re-analyze using the conventional protocol of *Myotis muricola* from Thailand.

Table 1. Karyotype reviews in the genus *Myotis* (Chiroptera: Vespertilionidae)

Species	2*n*	NFa	X	Y	Karyotype	NORs	Locality	Reference
Myotis albescens	44	50	m	Un	6m+2sm+34t	-	Peru	Bickham *et al.* (1986)
M. altarium	44	50	sm	a	8m/sm+34t	-	China	Wu *et al.* (2006)
M. annectans	46	52	sm	a	6m+2sm+36t	-	Thailand	Bickham *et al.* (1986)
M. ater	44	50	sm	-	6m+2sm+34t	-	Thailand	Bickham *et al.* (1986)
M. bechsteinii	44	52	m	sm	8m+-4sm/a+ 30t	-	Turkey	Karataş *et al.* (2013)
M. brandtii	44	50	m	t	8m+34t	-	Turkey	Karataş *et al.* (2013)
M. chinensis	44	50	sm	a	8m/sm+34t	-	China	Wu *et al.* (2006)
M. emarginatus	44	50	m	a	6m+ 2sm+34t	4	Turkey	Albayrak *et al.* (2020)
M. horsfieldi	44	50	sm	a	8m/sm+34t	-	China	Wu *et al.* (2009)
	44	52	sm	a	6m+4sm+32t	-	Thailand	Supanuam *et al.* (2013)
M. keaysi	44	50	sm	a	6m+2sm+34t	-	Costa Rica	Bickham *et al.* (1986)
M. lucifugus	44	50	sm	Un	6m+2sm+34t	-	Canada	Bickham *et al.* (1986)
M. muricola	44	50	sm	-	6m+2sm+34t	-	Thailand	Bickham *et al.* (1986)
	44	50	sm	t	6m+2sm+34t	2	Thailand	The present study
M. myotis	44	50	m	a	6m+2sm+34t	2	Turkey	Baydemir (2015)
M. mystacinus	44	52	sm	a	6m+4sm+32t	-	Thailand	Harada et al (1985)
M. ricketti	46	52	sm	a	8m/sm+36t	-	China	Wu *et al.* (2006)
M. siligorensis	44	52	sm	a	6m+4sm+32t	-	Thailand	Harada *et al.* (1985)

Abbreviations: 2*n* = diploid chromosome number, NFa = fundamental number of autosome (number of autosome arms), NORs = nucleolar organizer regions, m = metacentric, sm = submetacentric, a = acrocentric, t = telocentric chromosome, Un = unidentified chromosome, and-= not available.

2. Materials and methods

2.1 Sample collection and chromosome preparation

Five male and five female specimens of *M. muricola* (Figure 1) were collected from Maha Sarakham province, Northeastern Thailand. All Nepalese whiskered bats were transferred to the laboratory and kept under standard conditions for one day prior to the experimentation. The study was undertaken following ethical protocols. All animals were treated according to the rules of the Thai institutional animal care and use committee (Permit No. U1-05822-2559).

Chromosomes were directly prepared *in vivo* (Ota *et al.*, 1990; Donbundit *et al.*, 2020) by injecting 0.1% colchicine into the bats' intramuscular and abdominal cavity and then left for 12 hours. Samples of bone marrow (in males and females) were cut into small pieces and then mixed with 0.075 M potassium chloride (KCl). After discarding all large cell pieces, 15 mL of cell suspension was transferred to a centrifuge tube and incubated for 30-40 minutes, then centrifuged at 3,000 rpm for 8 minutes. The cell suspension was fixed in fresh cool fixative of methanol:glacial acetic acid (3:1) and gradually made up to 8 mL before centrifuging again at 3,000 rpm for 8 minutes, whereupon the supernatant was discarded. Fixation was repeated until the supernatant was clear and the pellet was mixed with 1 mL fixative.

Figure 1 General characteristics of *M. myotis* from Thailand. Scale bar = 3 cm.

2.2 Chromosome staining

Conventional staining and Ag-NOR banding techniques were performed as follows. A drop of the mixture was added to a clean and cold slide by micropipette, followed by the air-dry technique. The slide was conventionally stained with 20% Giemsa solution for 30 minutes (Patawang *et al.*, 2014; Sarasan *et al.*, 2021). Then, the slides were rinsed thoroughly with running tap water to remove excess stain.

Two drops of 2% gelatin and four drops of 50% silver nitrate were put on slide, respectively. Then it was sealed with cover glass and incubated at 60 °C for 5-10 minutes. After that, it was soaked in distilled water until the cover glass was separated (Howell & Black 1980).

2.3 Chromosome analysis

Ten clearly observable metaphase cells with well spread chromosomes of each male and female were selected and photographed. The lengths of short arm chromosome (Ls) and long arm chromosome (Ll) were measured and the length of total arm chromosome (LT = Ls+Ll) was calculated. The relative length (RL), the centromeric index (CI) and standard deviation (SD) of RL and CI were analyzed according to the chromosome classification of Chaiyasut (1989) and Turpin and Lejeune (1965). Chromosome types were described as metacentric (m), submetacentric (sm), acrocentric (a) and telocentric (t) chromosomes, respectively. The Fundamental Number (NF, number of chromosome arms) was obtained by assigning a value of two to metacentric, submetacentric and acrocentric chromosomes and one to telocentric chromosomes. All parameters were used in generating karyotypes and ideograms.

2.4 Fluorescence *in situ* hybridization (FISH)

The use of microsatellite probes described by Kubat *et al.* (2008) was followed here with slight modifications. The microsatellite probes: $d(GC)_{15}$ and $d(CGG)_{10}$ were directly labeled with Cy3 at the 5´-terminal during synthesis by Sigma (St. Louis, MO, USA). Fluorescence *in situ* hybridization (FISH) was performed under highly stringent conditions on mitotic chromosome spreads (Pinkel *et al.*, 1986). After denaturation of chromosomal DNA in 70% formamide/2×SSC (saline sodium citrate) at 70 °C, spreads were incubated in 2×SSC for 4 minutes at 70 °C. The hybridization mixture (2.5 ng/μL each probe, 2 μg/μL salmon sperm DNA, 50% deionized formamide, 10% dextran sulfate) was dropped on the slides and the hybridization was performed overnight at 37 °C in a moist chamber containing 2×SSC. The post hybridization wash was carried out with 1× SSC for 5 minutes at 65 °C. A final wash was performed at room temperature in 4×SSCTween for 5 minutes. Finally, the chromosomes were counterstained with DAPI (1.2 μg/mL), mounted in antifading solution (Vector, Burlingame, CA, USA) and analyzed in fluorescence microscope Nikon ECLIPSE.

3. Results and discussion

3.1 Diploid chromosome number (2*n*), fundamental number (NF) and karyotype

The conventional Giemsa staining karyotype of *M. muricola* specimens from Maha Sarakham Province shows $2n = 44$ with NF = 53 in male and 54 in female (Figure 2). The types of autosomes are six large metacentrics, two small submetacentrics, 34 small telocentric chromosomes and one pair of sex chromosomes (X, Y). The X is a large-sized metacentric chromosome. The small-sized Y chromosome is telocentric. The karyotype formula of *M. muricola* is as follows:

$2n$ (44) = L^{m}_{6}+ S^{sm}_{2}+ S^{t}_{34}+ sex chromosomes

The diploid chromosome number ($2n$) and fundamental number of autosome (NFa) of *M. muricola* from Maha Sarakham Province, Northeastern Thailand are 42 and 50, respectively, which do not differ from the conspecific populations in Huai Kha Khang Wildlife Sanctuary, Uthai Thani Province, Central Thailand (Bickham *et al.*, 1986). The karyotype of *M. muricola* in this study presents high conservation and there is no additional translocation. Previous reports described the autosomal complement of the species ($2n = 44$, NFa = 50) as three large pairs and a small pair of bi-arm chromosomes, and 17 mono-arm chromosome pairs ranging in size from medium to small (Harada *et al.*, 1985; Bickham *et al.*, 1986; Wu *et al.*, 2006; 2009; Karataş *et al.*, 2013; Supanuam *et al.*, 2013; Baydemir, 2015; Albayrak *et al.*, 2020). The sex chromosomes were characterized as an average submetacentric chromosome (X) and a small chromosome acrocentric (Y) (Faria & Ribeiro, 2018). Such karyotype was delineated for another 15 species of the genus (Table 1).

Although *Myotis* is considered as one of the most diverse genera (Simmons, 2005), the most common diploid number of $2n = 44$ is found in most examined species and 25 other vespertilionid species (e.g., Volleth & Heller, 1994). Their karyotypes are generally considered as primitive for the family Vespertilionidae (Bickham, 1979: Bickham *et al.*, 2004). According to morphological characteristics, *Myotis* appears to be the most primitive genus of Vespertilionidae. It retains the maximum number of teeth known in the order and has the slightest possible degree of special modification in external form (Miller, 1907; Bickham *et al.*, 2004).

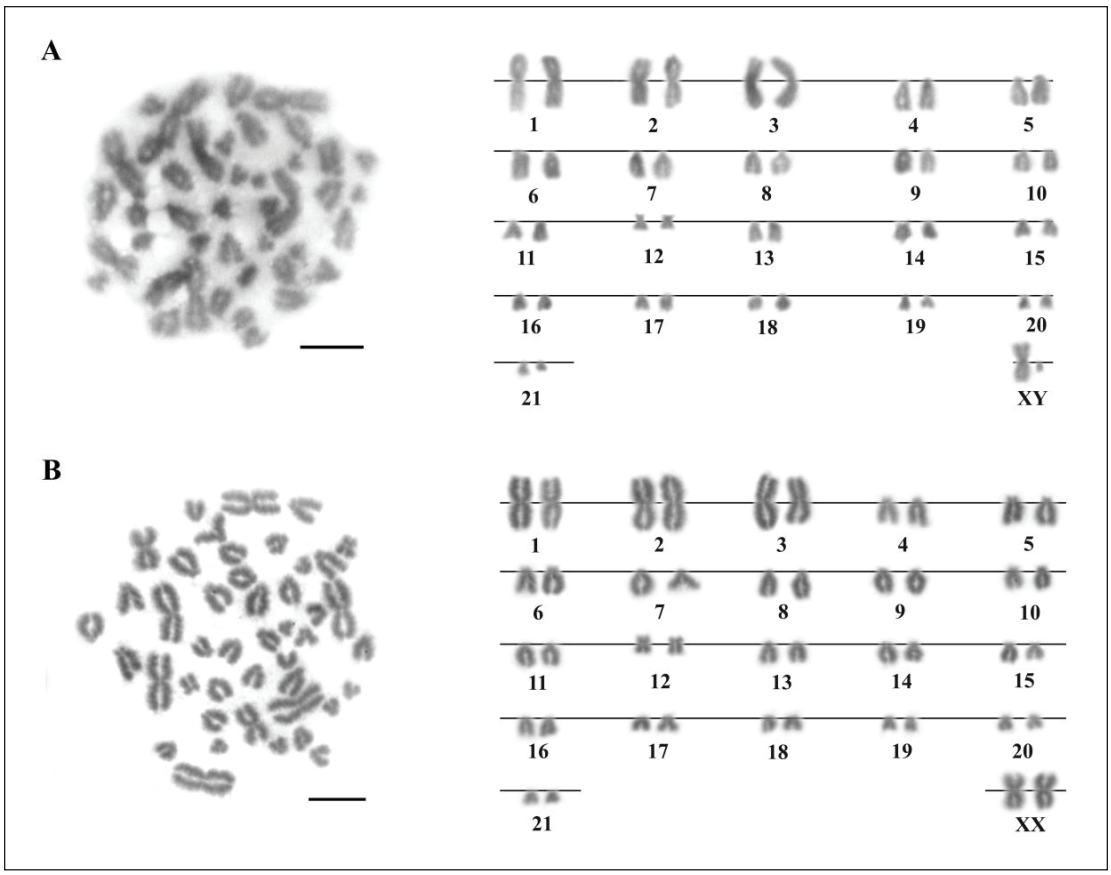

Figure 2. Metaphase chromosome plates and karyotypes of *M. muricola* (A) male (B) female by conventional Giemsa technique. Scale bars = 5 μm.

3.2 Nucleolar organizer region (NOR)

The objective of Ag-NOR staining technique is to detect NORs region, which represent the location of genes that function in ribosome synthesis (18S and 28S ribosomal RNA). NORs produce numerous gene expressions and comprise non-histone proteins more than other chromosome regions. Accordingly, the specific dark band (NOR positive) is induced by the reduction of organic silver by these proteins that change silver to be dark (Sharma *et al.*, 2002). The pattern of NORs in the karyotype of female *M. muricola* is described for the first time by this technique. We found the observable NORs as well as secondary constrictions on the regions adjacent to the centromeres of the long arm of telocentric chromosome pairs 8th and 16th (Figure 3). Gorobeyko and Kartavtseva (2018) reported that the species of the genus *Myotis* showed the centromeric NORs with the distributional pattern of NORs in *Myotis* karyotype being species-specific characteristics.

Species of the genus *Myotis* usually possessed more significant number of NOR-bearing autosomes (Volleth, 1987). Several species demonstrated the distributed NORs in the karyotype of this genus (Volleth, 1987, Volleth & Tidemann, 1989; Volleth & Heller, 2012; Albayrak *et al.*, 2020).

This finding of only two NOR sites in *M. muricola* from Thailand is exceptional for the purpose. Indeed, the presence of NORs indicated the location of the 18S ribosomal RNA gene as active NOR implying the higher chromosome evolution. In addition, this information can be used for further evolutionary studies and classification.

The ideogram shows a continuous length gradation of chromosomes. All parameters of chromosome measurement on mitotic metaphase cells (from all specimens) are illustrated in Table 2. Ideogram by conventional Giemsa staining is shown in Figure 4.

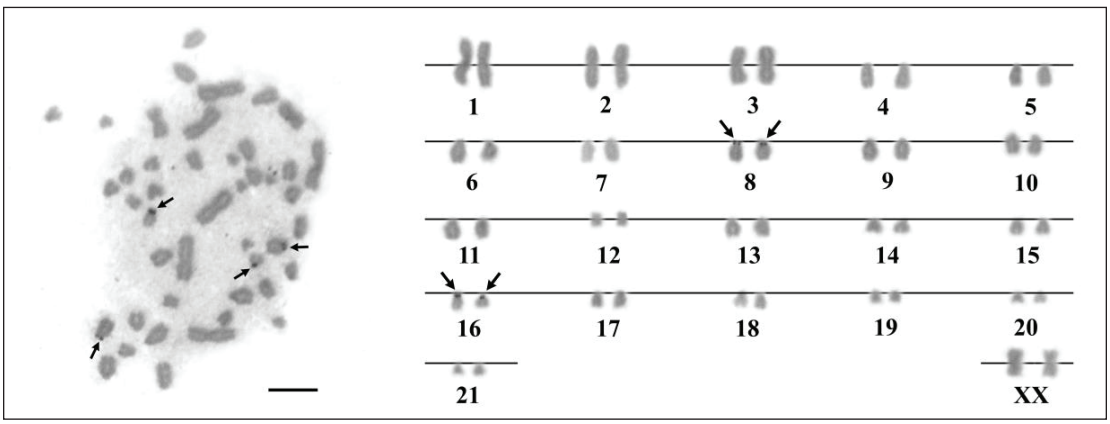

Figure 3. Metaphase chromosome plate and karyotype of *M. muricola* (female) by Ag-NOR staining technique. Arrows indicate nucleolar organizer regions (NORs). Scale bar = 5 μm.

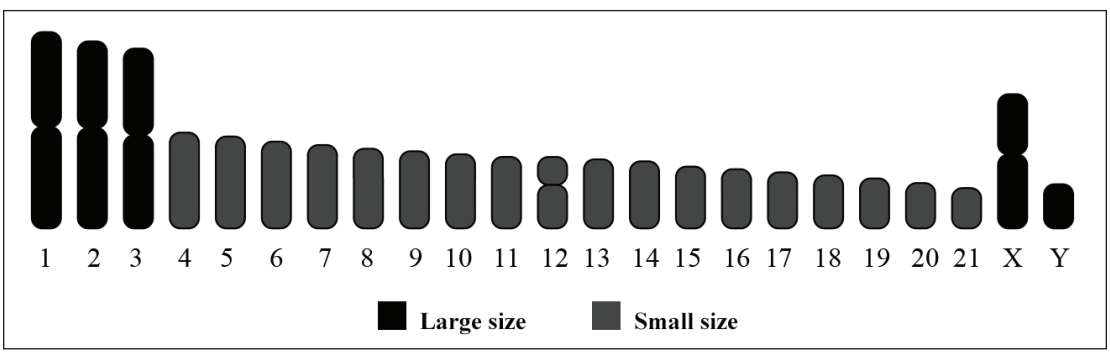

Figure 4. Ideogram of *M. muricola* representing lengths, shapes of chromosomes and the haploid chromosome set (*n*) by conventional Giemsa staining technique.

Table 2. Mean length of short arm chromosome (Ls), long arm chromosome (Ll), length of total chromosomes (LT), centromeric index (CI), relative length (RL) and standard deviation (SD) of CI and RL from 20 metaphases of male and female Nepalese whiskered myotis (*Myotis muricola*) 2*n* = 44.

Pair	Ls (µm)	Ll (µm)	LT (µm)	RL ± SD	CI ± SD	Size	Type
1	2.766	2.956	5.722	0.101±0.006	0.517±0.027	Large	Metacentric
2	2.526	2.925	5.450	0.097±0.005	0.538±0.031	Large	Metacentric
3	2.522	2.720	5.242	0.093±0.004	0.520±0.016	Large	Metacentric
4	0.000	2.793	2.793	0.050±0.002	1.000±0.000	Small	Telocentric
5	0.000	2.679	2.679	0.048±0.002	1.000±0.000	Small	Telocentric
6	0.000	2.530	2.530	0.045±0.002	1.000±0.000	Small	Telocentric
7	0.000	2.430	2.430	0.043±0.002	1.000±0.000	Small	Telocentric
8*	0.000	2.355	2.355	0.042±0.002	1.000±0.000	Small	Telocentric
9	0.000	2.250	2.250	0.040±0.002	1.000±0.000	Small	Telocentric
10	0.000	2.161	2.161	0.038±0.002	1.000±0.000	Small	Telocentric
11	0.000	2.085	2.085	0.037±0.002	1.000±0.000	Small	Telocentric
12	0.815	1.268	2.083	0.037±0.004	0.605±0.047	Small	Submetacentric
13	0.000	2.013	2.013	0.036±0.002	1.000±0.000	Small	Telocentric
14	0.000	1.956	1.956	0.035±0.002	1.000±0.000	Small	Telocentric
15	0.000	1.802	1.802	0.032±0.002	1.000±0.000	Small	Telocentric
16*	0.000	1.727	1.727	0.031±0.002	1.000±0.000	Small	Telocentric
17	0.000	1.635	1.635	0.029±0.002	1.000±0.000	Small	Telocentric
18	0.000	1.545	1.545	0.028±0.002	1.000±0.000	Small	Telocentric
19	0.000	1.457	1.457	0.026±0.003	1.000±0.000	Small	Telocentric
20	0.000	1.322	1.322	0.024±0.002	1.000±0.000	Small	Telocentric
21	0.000	1.176	1.176	0.021±0.004	1.000±0.000	Small	Telocentric
X	1.757	2.154	3.910	0.070±0.007	0.533±0.044	Large	Metacentric
Y	0.000	1.289	1.289	0.017±0.002	1.000±0.000	Small	Telocentric

Remark: * = NOR-bearing chromosomes

3.3 Patterns of microsatellite repeats

Microsatellites or simple sequence repeats (SSRs) are oligonucleotides of 1-6 base pairs in length, forming excessive tandem repeats of usually 4 to 40 units (Tautz & Renz, 1984; Ellegren, 2004; Chistiakov *et al.*, 2006). They show ample distribution throughout eukaryotic genomes, scattered or clustered in euchromatin and heterochromatin. They are highly polymorphic regarding copy number deviation (Ellegren, 2004). The mapping of microsatellite repeats on the female's chromosomes of *M. muricola* represents the $d(GC)_{15}$ repeat showing specifically on chromosome pairs 1, 4 and

10 (Figure 5A), while the hybridization signal of d(CGG)$_{10}$ repeat was distributed throughout the whole chromosomes

(Figure 5B). Our finding of microsatellite patterns of *M. muricola* revealed for the first time that can be used as diagnostic feature as well as marker chromosome.

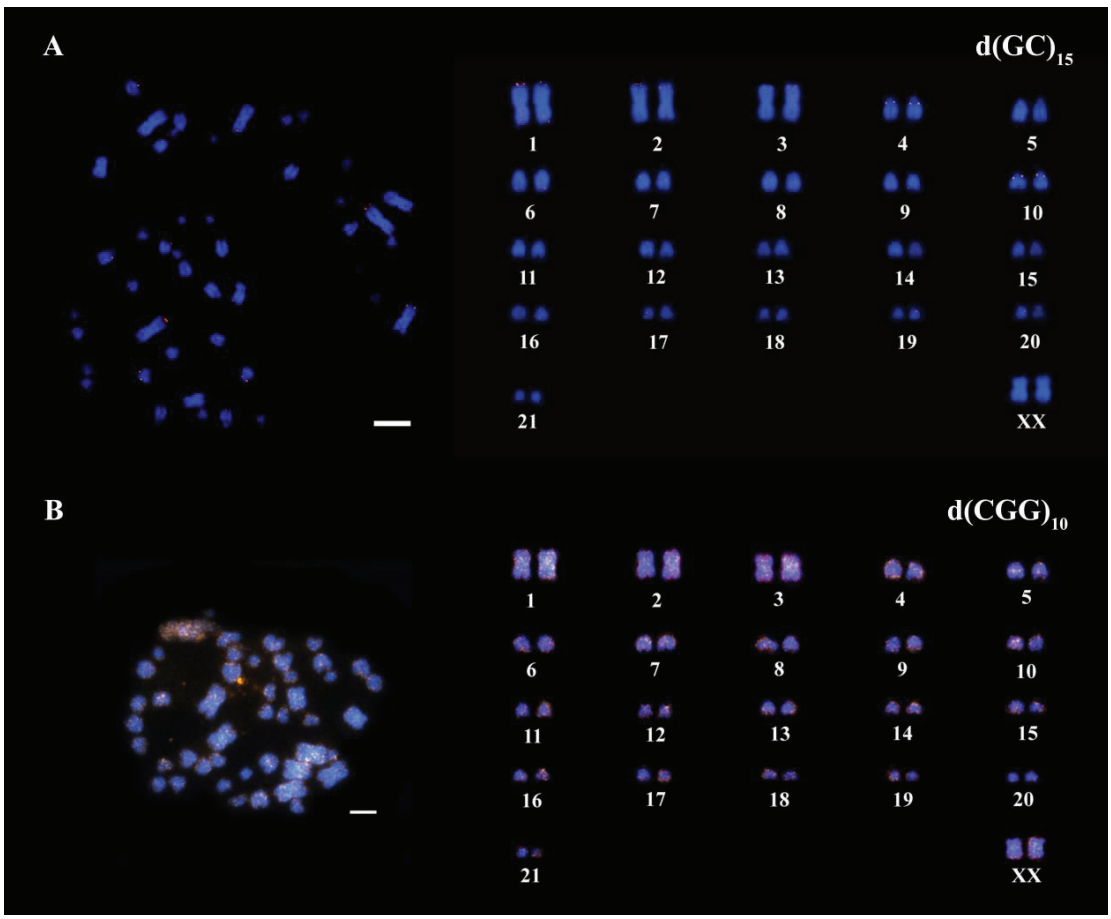

Figure 5. Metaphase chromosome plates and karyotypes of *M. muricola* (female) by FISH technique presenting the patterns of microsatellite d(GC)$_{15}$ (A) and d(CGG)$_{10}$ (B). Scale bars = 5 µm.

4. Conclusion

The present research is the first report on NOR and microsatellites d(GC)$_{15}$ and d(CGG)$_{10}$ mapping in *M. muricola*. The results obtained here of diploid chromosome number, the fundamental numbers, karyotypes, pairs having NORs and patterns of microsatellite distributions on chromosomes can be used to support the further investigation on taxonomy, biodiversity conservation, chromosome evolution and evolutionary relationship among the genus *Myotis* and others.

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