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Cytogenetic Analysis of the Variable Squirrel (*Callosciurus finlaysonii* Horsfield, 1824) by Conventional Staining, Ag-NOR Staining and Fluorescence in situ Hybridization Techniques

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ABSTRACT

Variable Squirrel belong to the genus *Callosciurus* which has several color patterns as well as and difficult to classify from other species. Cytogenetics is the study of chromosomal features as useful tool on taxonomic and evolutionary studies of squirrel. Moreover, there are scarcely records of cytogenetics of this squirrel. Therefore, the purpose of the present study are to establish karyotype and ideogram, to investigate the NOR site and the pattern of microsatellite sequence $(GC)_{15}$ and $(CGG)_{10}$ using convention staining, Ag-NOR staining, and fluorescence in situ hybridization techniques. The result showed that the diploid chromosome (2n) of *C. finlaysonii* was 40, the fundamental number was 74 in both sexes, and the sex determination was XY sex-determination system. The karyotype was composed of 4 large metacentric, 8 large submetacentric, 8 large acrocentric, 2 medium submetacentric, 2 medium acrocentric, 2 medium telocentric, 4 small metacentric, 2 small submetacentric, 2 small acrocentric and 4 small telocentric chromosomes. X chromosome was medium metacentric and Y chromosome was small metacentric chromosomes. NOR's were located on

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chromosome pairs 14, and microsatellite $(GC)_{15}$ and $(CGG)_{10}$ repeats were highly scattered of all chromosome pairs throughout whole genome. This result was able to study evolutionary of squirrel's chromosome compare with the ancestors. The karyotype formula was 2n (40) = $L^{m}{}_{4} + L^{sm}{}_{8} + L^{a}{}_{8} + M^{sm}{}_{2} + M^{a}{}_{2} + M^{t}{}_{2} + S^{m}{}_{4} + S^{sm}{}_{2} + S^{a}{}_{2} + S^{t}{}_{4}$ + sex chromosome. This cytogenetic data provides useful basic information for further study on the comparison of evolutionary relationship and cytotaxonomy in the genus *Callosciurus*.

INTRODUCTION

Around the world, including Thailand, there are many different types of mammals. Squirrels are one of the most common mammals. In the Rodentia Order, the Sciuridae family can be divided into 2 subfamilies: Pteromyinae and Sciurinae. Squirrels are wild animals that can be seen easily for example, it can be found in forest areas and population distribution throughout Thailand, especially *Callosciurus finlaysonii* is a fairly versatile squirrel (1). *C. finlaysonii* is a squirrel native to Thailand, Myanmar, Laos, Cambodia and Vietnam. It has been introduced to Italy (2) The *C. finlaysonii* belong to the Sciurinae and have 13 subspecies (3). It has big eyes, large upper and lower teeth like each pair, and have hair covering the entire body. In general, the color of the coat can be found in a wide variety. For example, whole body cream color, cream color on the abdomen and there are other parts in black color (4). Cytogenetics is the study of traits properties and behavior of chromosomes during the dividing body cells for growth, or mitosis and gamete meiosis division, including study of factors affecting chromosomal changes (5). Currently, it is reported that squirrels of the genus *Collosciurus* have chromosomes 38-40 (6) and colored squirrels (*C. finlaysonii*) have 40 chromosomes. (7). Although *C. finlaysonii* are abundant in Thailand, the cytogenetics are studied and some of the information was reported but relatively little. Therefore, the objectives of this research are to study chromosomes, nucleolar organizer region (NOR), karyotype, ideogram and the microsatellite $(GC)_{15}$, and (CGG_{10}) patterns that remarkably presented on the *C. finlaysonii* chromosomes. The study moves forward our understanding of both the karyotype evolution mechanisms and speciation in the genus *Callosciurus*, and increases the knowledge available for implementation of polyploidy manipulation, hybridization, sex control, and other potential genetic improvements in the future.

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MATERIALS AND METHODS

Sample collection

Samples of 3 male and 3 female squirrels were collected by placing a mousetrap cage on a tree at the junction between SC03 and SC07 buildings, Khon Kaen University, Thailand.

Chromosome preparation, Giemsa's staining and AgNORs banding technique

Metaphase chromosomes were directly prepared in vivo as following (8) and (9). Subsequently, chromosomes were stained with 20% Giemsa solution and 50% silver nitrate for Ag-NOR banding (10).

Chromosome checking

Twenty metaphases of each specimen were selected and photographed. The length of the short arm chromosome (Ls) and the long arm chromosome (Ll) were measured from 20 perfect metaphase plates of each sex, while the length of the total arm chromosome (LT) was calculated (LT = Ls + Ll). The relative length (RL), the centromeric index (CI), and standard deviation (SD) of RL and CI were estimated. The CI (q/p + q) between 0.50-0.59, 0.60-0.69,0.70-0.89, and 0.90-1.00 are described as metacentric (m), submetacentric (sm), acrocentric (a), and telocentric (t) chromosomes, respectively. The fundamental number (NF) was obtained by assigning a value of 2 to the m, sm and a chromosomes and 1 to the t chromosomes. All data were used in karyotyping and diagramming (11).

Fluorescence in situ hybridization (FISH)

FISH was performed on metaphase chromosome spreads with specific probes bind to the target DNA location (12). Both rDNA probes were directly labeled with the Nick-translation Labeling Kit (Jena Bioscience, Jena, Germany), using the fluorescent labels Atto488 (18S rDNA) and Atto550 (5S rDNA), according to the manufacturer's manual (13). The using of microsatellites $d(GC)_{15}$, and $d(CGG)_{10}$ probes described by (14) was followed with slight modifications. Sequences were directly labeled with Cy3 at 5′ terminals during synthesis by Sigma (St. Louis, MO, USA). FISH was performed on mitotic chromosome spreads (15) under highly stringent conditions, as previously reported. The evaluation was carried out on an epifluorescence microscope Olympus BX50 (Olympus Corporation, Ishikawa, Japan).

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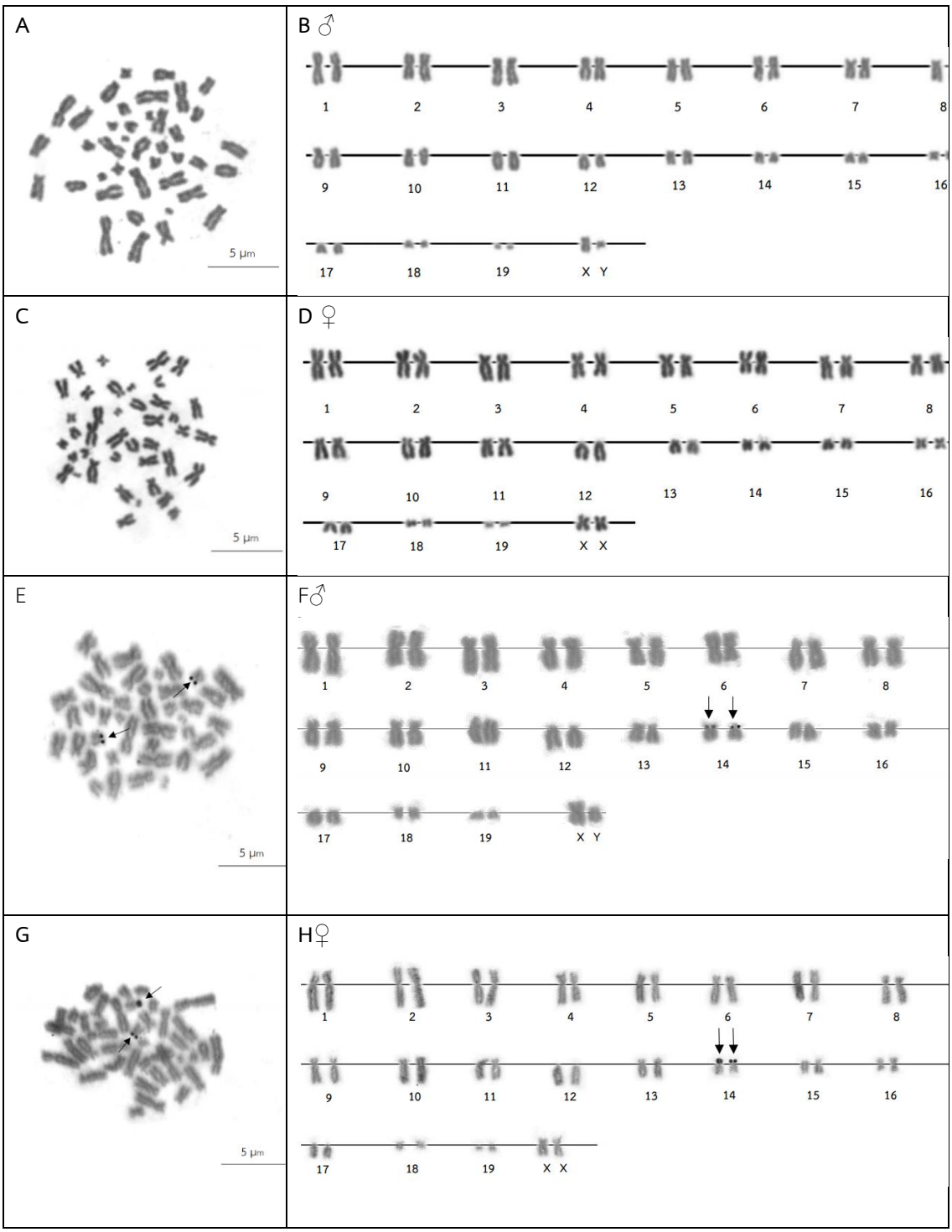

Figure 1 Metaphase chromosome plates and karyotypes of male (A-B and E-F) and female (C-D and G-H), squirrel (*Callosciurus finlaysonii* Horsfield, 1824), 2n=40 by conventional straining (A-D) and Ag-NOR banding (E-H) technique. Scale bars indicate 5µm. The arrows indicate nucleolar organizer regions (NOR)

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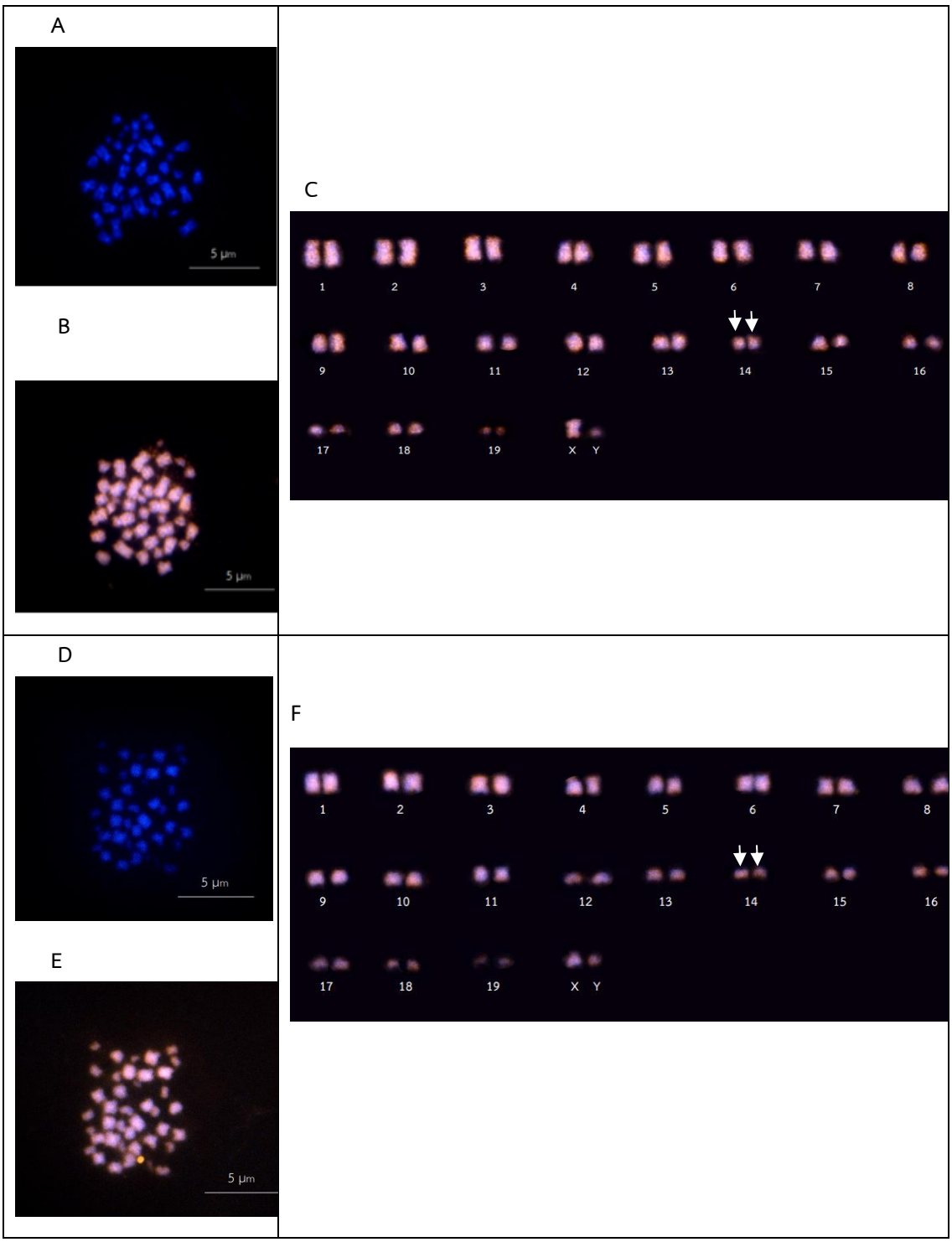

Figure 2 Karyotype of squirrel (*Callosciurus finlaysonii* Horsfield, 1824) 2n=40 (C, F) arranged from chromosomes after double-fluorescence in situ hybridization (FISH) with 5S rDNA (red) and 18S rDNA (green) probes (A-C), FISH with $d(GC)_{15}$, (A-B) FISH with $d(CGG)_{10}$ (D-F). Bars indicate 5 µm. The arrows indicate probe signals

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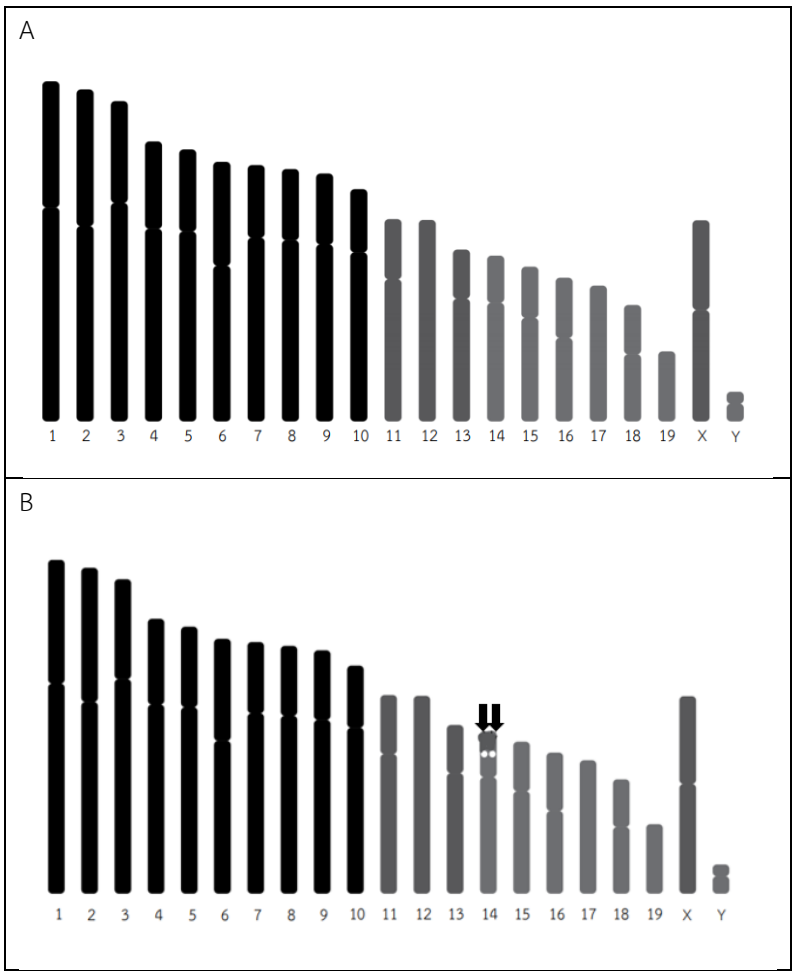

Figure 3 Idiograms of the squirrel (*Callosciurus finlaysonii* Horsfield, 1824), representing the haploid set (n) by conventional staining (A) and Ag–NOR banding techniques (B). Arrows indicate NOR site.

Table 1 Cytogenetics review in the genus *Callosciurus*

Species	2n	NF	Karyotype	Sex system	NOR	Locality	Reference
C. finlaysonii	40	78	8m+4sm+20a+6t	X(m), Y(m)	-	Thailand	Tanomtong *et al*., (2009)
C. finlaysonii	42	74	12m+20sm+6a	X(sm), Y(sm)	-	Thailand	Usa pasuk (2010)
C. finlaysonii	40	-	12m+20sm+6a	X, Y	-	Malaysia Philippines Indonesia	Nedler *et al*., (1975)
C. finlaysonii	40	-	8m+4sm+20a+6t	X(m), Y(m)	-	Thailand	Pinthong *et al*., (2016)

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Species	2n	NF	Karyotype	Sex system	NOR	Locality	Reference
C. erythraeus	40		12m+14sm+6a+6t	X, Y	-	Thailand	Pinthong *et al*., (2016)
C. caniceps	40		12m+8sm+14a+6t	X, Y	-	Thailand	Pinthong et al., (2016)
C. caniceps	40	68	-	X, Y	16	ASEAN countries	Oshida and Yoshida, (1999)
C. flavimanus	40	72	-	X, Y	15	ASEAN countries	Oshida and Yoshida, (1999)
C. eryrhraeus	40	70	-	X, Y	15	ASEAN countries	Oshida and Yoshida, (1999)
C. nigrovittatus	40	70		X, Y	16	ASEAN countries	Oshida and Yoshida, (1999)
C. notatus	40	68		X, Y	16	ASEAN countries	Oshida and Yoshida, (1999)
C. prevostii	40	70		X, Y	16	ASEAN countries	Oshida and Yoshida, (1999)
C. erythraeus	40	-		X, Y	-	ASEAN countries	Oshida and Yoshida, (1999)
C. prevostii	40	-		X, Y	-	ASEAN countries	Oshida and Yoshida, (1999)
C. albescens	38	-		X, Y	-	ASEAN countries	Oshida and Yoshida, (1999)

Remarks: 2n = diploid chromosome number, NF = fundamental number, NOR = nucleolar organizer regions, m = metacentric chromosome, sm = submetacentric chromosome, a = acrocentric chromosome and t = telocentric chromosome

Table 2 Means of the short arm length (Ls), long arm length (Ll) and total arm length of chromosomes (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL and CI of 20 metaphase cells of the male and female (*Callosciurus finlaysonii*), 2n=40

Chro. pair	LS	LL	LT	RL±SD	CI±SD	Chro. Size	Chro. Type
1	1.540	2.613	4.154	1.732 ± 0.278	0.631 ± 0.040	Large	Submetacentric
2	1.669	2.385	4.054	1.704 ± 0.297	0.583 ± 0.019	Large	Metacentric
3	1.243	2.669	3.912	1.642 ± 0.292	0.674 ± 0.047	Large	Submetacentric
4	1.066	2.353	3.419	1.445 ± 0.229	0.678 ± 0.051	Large	Submetacentric

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Chro. pair	LS	LL	LT	RL±SD	CI±SD	Chro. Size	Chro. Type
5	1.001	2.320	3.321	1.387 ± 0.210	0.696 ± 0.051	Large	Submetacentric
6	1.261	1.874	3.135	1.330 ± 0.234	0.591 ± 0.048	Large	Metacentric
7	0.886	2.244	3.131	1.309 ± 0.196	0.715 ± 0.040	Large	Acrocentric
8	0.869	2.213	3.081	1.289 ± 0.203	0.716 ± 0.039	Large	Acrocentric
9	0.866	2.161	3.027	1.261 ± 0.209	0.714 ± 0.031	Large	Acrocentric
10	0.772	2.064	2.835	1.191 ± 0.209	0.722 ± 0.028	Large	Acrocentric
11	0.734	1.736	2.470	1.065 ± 0.231	0.695 ± 0.050	medium	Submetacentric
12	0.000	2.460	2.460	1.025 ± 0.221	1.000 ± 0.000	medium	Telocentric
13	0.599	1.499	2.098	0.877 ± 0.161	0.711 ± 0.028	medium	acrocentric
*14	0.575	1.449	2.025	0.847 ± 0.144	0.713 ± 0.019	small	acrocentric
15	0.616	1.273	1.889	0.781 ± 0.163	0.680 ± 0.050	small	Submetacentric
16	0.726	1.028	1.754	0.739 ± 0.121	0.580 ± 0.034	small	Metacentric
17	0.000	1.658	1.658	0.6914 ±0.1175	1.000 ± 0.000	small	Telocentric
18	0.592	0.829	1.420	0.598 ± 0.104	0.576 ± 0.026	small	Metacentric
19	0.000	0.864	0.864	0.360 ± 0.063	1.000 ± 0.000	small	Telocentric
X	1.092	1.364	2.456	0.996 ± 0.368	0.578 ± 0.034	medium	Metacentric
Y	0.146	0.216	0.361	0.156 ± 0.166	0.578 ± 0.021	small	Metacentric

Remarks: Chro.: Chromosome, *: NOR-bearing chromosome

RESULTS AND DISCUSSION

Diploid chromosome number (2n), fundamental number (NF) and karyotype of Callosciurus finlaysonii

The model diploid number of *C. finlaysonii* was 2n = 40 chromosomes and the fundamental number was 74 (NF = 74) in both male and female. The sex determination system was XY with the karyotype formula 2n (40) = $L^{m}_{4} + L^{sm}_{8} + L^{a}_{8} + M^{sm}_{2} + M^{a}_{2} + M^{t}_{2} + S^{m}_{4} + S^{sm}_{2} + S^{a}_{2} + S^{t}_{4}$ + Sex Chromosome.

Chromosome type and size of C. finlaysonii

The karyotype was composed of 4 large metacentric, 8 large submetacentric, 8 large acrocentric, 2 medium submetacentric, 2 medium acrocentric, 2 medium telocentric, 2 small metacentric, 2 small submetacentric, 2 small acrocentric and 4 small telocentric chromosomes. The X chromosome was medium metacentric and Y chromosome was small metacentric chromosomes (Figure 1). All parameters of chromosome are shown in Table 2.

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Chromosome marker of *C. finlaysonii*

The determination of a chromosome marker for this species was firstly obtained by Ag-NOR staining. The nucleolar organizer regions (NORs) were mapped to interstitial small acrocentric of the short arm of the chromosome pairs 14. (Figure 3).

Patterns of microsatellite d(GC)$_{15}$ and d(CGG)$_{10}$ repeats in C. finlaysonii

The mapping of microsatellite repeats on the chromosomes of *C. finlaysonii* showed that (GC)$_{15}$ and (CGG)$_{10}$ signals were observed on all chromosome pairs. These signals were distributed throughout the whole chromosomes. (Figure 2. A-F).

Idiograms of C. finlaysonii chromosomes

All previous chromosomal studied results were summarized (Table 1). The idiograms presenting shapes, sizes and probe signals on the chromosomes of *C. finlaysonii* are shown in Figure 3.

CONCLUSION

The result showed that the diploid chromosome (2n) of *C. finlaysonii* was 40, the fundamental number was 74 in both sexes, and the sex determination was XY sex-determination system. The karyotype was composed of 4 large metacentric, 8 large submetacentric, 8 large acrocentric, 2 medium submetacentric, 2 medium acrocentric, 2 medium telocentric, 4 small metacentric, 2 small submetacentric, 2 small acrocentric and 4 small telocentric chromosomes. X chromosome was medium metacentric and Y chromosome was small metacentric chromosomes. Which the study results are consistent with the research of (20) and from the analysis of the chromosome type in each rod, it was found that there are types of chromosomes 4, 5, 11, 15 different from the research of (22). It has been reported that the chromosome rods are acrocentric type, respectively but the results this research we are found submetacentric type. Which in this research paper used a total of 15 cells and in this research used 20 cells or it may be variation at the subspecies level. (16). The determination of a chromosome marker for this species was firstly obtained by Ag-NOR staining. The nucleolar organizer regions (NORs) were mapped to interstitial small acrocentric of the short arm of the chromosome pairs 14. This is consistent with the research of (16). It was reported was found on the short arm of the acrocentric type chromosome. The 14 pair of acrocentric can be used as a chromosome marker of variable squirrel because, It has a different position than squirrels of the genus *Callosciurus*, as reported in research by (17). As reported in the research of (18), *Callosciurus canicep*, *C. flavimanus*, *C. erythraeus*, *C. nigrovittatus*, *C. notatus*, *C. prevostii* had the NOR position on the chromosome pair 16, 15, 15, 16, 16, and 16, respectively. The reason why variable squirrels in the same genus have different NOR positions may be caused by a change in the shape of chromosome translocation, which is the fracture and connection of an ancestor chromosome to form a living organism (19).

In summary, although variable squirrel are abundant in Thailand. And studies of cytogenetics some of the information on others is reported relatively litter. Which the data obtained from the study can lead to information for use in the classification or study of the chromosomal evolution of variable squirrel in the future.

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