การวิเคราะห์แคริโอไทป์ของอ้นเล็ก (*Cannomys badius*) ด้วยเทคนิคทางพันธุศาสตร์ระดับเซลล์แบบดั้งเดิมและระดับโมเลกุล

Karyological Analysis of Lesser Bamboo Rat, *Cannomys badius* (Rodentia, Rhizomyinae) by Classical and Molecular Cytogenetic Techniques

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บทคัดย่อ

การศึกษาพันธุศาสตร์ระดับเซลล์ของอ้นเล็ก (*Cannomys badius*) โดยเทคนิคแบบดั้งเดิมและเทคนิคฟลูออเรสเซนซ์อินไซทูไฮบริไดซ์เซชัน เก็บตัวอย่างจากจังหวัดเพชรบูรณ์ เตรียมโครโมโซมด้วยวิธีการเพาะเลี้ยงเซลล์เม็ดเลือดขาว เก็บเกี่ยวเซลล์ ย้อมสีแบบธรรมดาด้วยสีจิมซ่า ย้อมแถบสีแบบนอร์ และย้อมด้วยเทคนิคฟลูออเรสเซนซ์อินไซทูไฮบริไดซ์เซชัน ผลการศึกษาพบว่าอ้นเล็กมีจำนวนโครโมโซมดิพลอยด์ (2*n*) เท่ากับ 50 แท่ง โครโมโซมพื้นฐาน (NF) เท่ากับ 94 ออโตโซมประกอบด้วยโครโมโซมชนิดอะโครเซนทริกขนาดใหญ่ 12 แท่ง ซับเมทาเซนทริกขนาดกลาง 2 แท่ง อะโครเซนทริกขนาดกลาง 10 แท่ง เมทาเซนทริกขนาดเล็ก 4 แท่ง ซับเมทาเซนทริกขนาดเล็ก 6 แท่ง อะโครเซนทริกขนาดเล็ก 8 แท่ง และเทโลเซนทริกขนาดเล็ก 6 แท่ง โครโมโซมเอ็กซ์ เป็นชนิดเมทาเซนทริกขนาดใหญ่ พบตำแหน่งนอร์ 3 คู่ อยู่บนโครโมโซมคู่ที่ 7, 10 และ 17 จากการย้อมด้วยเทคนิคฟลูออเรสเซนซ์อินไซทูไฮบริไดซ์เซชัน ใช้โพรบไมโครแซทเทลไลท์ d(CA)$_{15}$ พบจุดสัญญาณติดบริเวณแขนข้างยาวใกล้กับเซนโทรเมียร์ของโครโมโซมคู่ที่ 13 ซึ่งข้อมูลนี้สามารถใช้เป็นเครื่องหมายทางพันธุกรรมสำหรับอ้นเล็กได้ และเป็นข้อมูลพื้นฐานไว้สำหรับการศึกษาขั้นสูงต่อไป

ABSTRACT

The lesser bamboo rat (*Cannomys badius*) from Phetchabun Province, Thailand was cytogenetically studied by classical and molecular cytogenetic techniques. Blood samples were taken from lesser bamboo rat and then subjected to standard whole blood T-Lymphocyte culture. The chromosomes were harvested by colchicine-hypotonic-fixation-air drying technique. Conventional, Ag-NOR banding and fluorescence *in situ* hybridization (FISH) techniques were used to apply on the metaphase chromosomes. The results showed that the karyotype of lesser bamboo rat had the diploid chromosome number of 2*n*=50 and the fundamental number (NF) of 94. The autosomes consisting of 12 large acrocentric, two medium submetacentric, 10 medium acrocentric, four small metacentric, six small submetacentric, eight small acrocentric and six small telocentric chromosomes. The X chromosome is the largest metacentric chromosome. The nucleolar organizer regions (NORs) were observed in the short arms of chromosome pairs 7, 10 and 17 for the first time. In addition, the molecular probe, microsatellite $d(CA)_{15}$ was revealed the hybridization signal detected at subcentromeric region in the long arms of chromosomes pair 13. The obtained results suggested that NORs and microsatellite $d(CA)_{15}$ probe can be used as specific markers for this species. Our results are also fulfilled the basic cytogenetic knowledge for *C. badius*.

คำสำคัญ: อ้นเล็ก (*Cannomys badius*) โครโมโซม แคริโอไทป์ พันธุศาสตร์โมเลกุล

Keywords: *Cannomys badius*, Chromosome, Karyotype, Molecular Cytogenetics

INTRODUCTION

The subfamily Rhizomyinae (Spalacidae, Rodentia) is known in the fossil record since the Oligocene. It is represented by three modern genera (*Rhizomys*, *Cannomys*, *Tachyorystes*). There are 17 species of rat and classified in two tribes (Rhizomyina, Trachyoryctini). The tribes Rhizomyini includes four species namely Hoary bamboo rat (*R. pruinosus*), Chinese bamboo rat (*R. sinensis*), large bamboo rat (*R. sumatrensis*), and lesser bamboo rat, *C. badius* (Hodgson, 1841) (Lekagul and McNeely 1988; Wilson and Cole, 2000; Parr, 2003; Musser and Carleton, 2005). The distributions of *C. badius* range from Eastern Nepal, through Northeast India, Bhutan, Southeastern Bangladesh, Myanmar, South China, Northwest Vietnam, Thailand and Cambodia. (Musser and Carleton, 2005)

The *C. badius* is a small, fairly stocky rodent that is smaller size distinguishes it from other bamboo rat species. It is covered with soft, dense, chestnut brown fur over the entire body, head and limbs. Some individuals show a vertical white streak of fur the snout up over the forehead. The cheeks are slightly paler brown. The comparatively short tail is virtually naked, covered in hairs and its footpads are smooth (Figure 1). Behavior of *C. badius* is commonly burrowing and habitat found in bamboo groves in sub-mountain and mixed deciduous forest of western, Northern Thailand (Lekagul and McNeely, 1988; Parr, 2003).

Although the animal cytogenetics has been studied so far, the reports of cytogenetic studies of bamboo rat are scarce. There are only few previous reports by Hsu and Johnson (1963) that reveals the diploid chromosome number of 2*n*=50 in male *R. sumatrensis* karyotype by conventional staining technique. The autosomes (24 pairs) compose of metacentric and submetacentric chromosomes. In

2013, Tanomtong et al. revealed the karyotype of the *R. pruinosus* from Thailand which had the diploid number 2*n*=50 and the fundamental number (NF) = 100 in both males and females. The numbers of bands in *R. pruinosus* are determined to be 234 and 280 by GTG-banding and high-resolution techniques, respectively. For the *C. badius* reported the standardization of karyotype, idiogram and described the chromosome banded by GTG-, CBG-, high-resolution techniques and Ag-NOR banding (Tanomtong et al., 2011). However, NORs could not be clearly observed (Table 1). In this study, we aim to confirm NORs and compare the results with previous reports. In addition, the molecular probe, microsatellite $d(CA)_{15}$ was used to detect if there is some specific hybridization pattern in *C. badius* that has not been studied yet. The results obtain can be fulfilled to the basic knowledge and accommodate for the further research.

Figure 1. General characteristic of the lesser bamboo rat, *C. badius*. Scale bar indicates 5 centimeters.

Table 1 Cytogenetic reviews of Asian bamboo rats in the subfamily Rhizomyinae (genera *Rhizomys* and *Cannomys*).

Species	2*n*	NF	NOR	G-	C-	FISH	Locality	Reference
R. pruinosus	50	-	-	-	-	-	Malaya	Hsu and Johnson (1963)
	50	100	+	+	+	-	Thailand	Tanomtong et al. (2013)
C. badius	50	94	-	+	+	-	Thailand	Tanomtong et al. (2011)
	50	94	+	-	-	+	Thailand	Present study

Remarks: 2*n* = diploid chromosome, NF = fundamental number (number of chromosome arms), NOR = Ag-NOR-banding, G- = G-banding, C- = C-banding, FISH = fluorescence *in situ* hybridization, + = available data and - = unavailable data.

MATERIALS AND METHODS

The sample collected from Petchabun Province, blood collections were taken from jugular vein of lesser bamboo rat and kept cooled in 10 mL heparinized vacuum tube to prevent blood clotting prior arriving to the laboratory. The lymphocytes were cultured using that of standard protocol (Rooney, 2001; Supanuam, et al. 2005; Jantarat, et al. 2007).

Cell culture

The RPMI 1640 medium was prepared with 1% PHA (Phytohemagglutinin) as a mitogen and kept in blood culture bottles of 5 mL each. The blood

samples of 0.4 mL were dropped into a medium bottle and mixed well. The culture bottle was loosely capped, incubate at 37 ºC under 5% of carbondioxide environment and regular shake in the morning and evening. When reaching harvest time at the 72 hour of incubation, Karyo Max Colcemid (10 µm/ml) was introduced and mixed well followed by further incubation for 20 minutes.

Cell harvest

The blood sample mixture was centrifuged at 1,600 rpm for 10 minutes and discarded supernatant. Adding 5 mL of hypotonic solution (0.075 M KCl) to the pellet and incubated for 30 minutes. The discarded supernatant and centrifugation at 1,600 rpm for 10 minutes. Cells were then fixed with fresh cold fixative consisting of methanol and glacial acetic acid (3: 1) by gradually added up to 5 mL, centrifuged at the same setting and discarded the supernatant. The fixative was repeated until the supernatant was clear. Finally, adding 1 mL of fixative to the pellet. The cell suspension was dropped onto a cleaned-cold slide by using micropipette and air-dry.

Chromosome stanning

Conventional staining, the slides were then applied with 20% Giemsa's solution for 30 minutes. Ag-NOR banding was applied 1 drop of 2% gelatin followed by 2 drops of 50% silver nitrate on the area of metaphase chromosome. The slides were then sealed with cover glasses and incubated at 60 degree Celsius for 5 minutes. After that washing the slides in distilled water until a coverslip was removed. (Howell and Black, 1980)

FISH was performed under high stringency conditions on mitotic chromosome spreads (Kubat et al. 2008). The metaphase chromosome slides were incubated with pepsin treatment (95 mL dH_2O + 5 mL 0.2N HCl) for 3 minutes at 37 degree Celsius. After denaturation of chromosomal DNA in 70% formamide at 83 degree Celsius. The hybridization mixture (2.5 ng/µl probes, 2 µg/µl DNA, 50% deionized formamide, 10% dextran sulphate) was dropped on the slides, and the hybridization was performed overnight at 37 degree Celsius in a moist chamber containing distilled water. The post hybridization wash was carried out with 4× SSC for 5 minutes at room temperature (2 times). Finally, the slides were counterstained with DAPI and mounted in an antifade solution (Vectashield from Vector laboratories).

FISH experiments with the microsatellites $d(CA)_{15}$ as probe were performed as described in Pinkel et al. (1986), with slight modifications. These sequences were directly labeled with Cy3 at 5′ terminal during synthesis by Sigma (St. Louis, MO, USA). 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.

RESULTS AND DISCUSSION

Cytogenetic study of *C. badius* using conventional, Ag-NOR banding and FISH techniques revealed that *C. badius* had the diploid chromosome number of 2*n*=50 that comprised 48 autosomes and two sex chromosomes (Figure 2A). The present results are accordance to the previous reports (Hsu and Johnson, 1963; Tanomtong et al., 2011; 2013) that the bamboo rat had 2*n*=50 in both genera.

The *C. badius* has the number of chromosome arm or fundamental number, NF=94, which according to the previous study reported by Tanomtong et al. (2011). However, in comparison to *R. pruinosus*, the karyotype of *C. badius* has NF less than *R. pruinosus* (Tanomtong et al., 2013). This fact suggests that some pericentric inversions have

occurred in the karyotype differentiation of this genus. In fact, the occurrence of chromosomal rearrangements has been considered a relatively common evolutionary mechanism inside the mammals (Shafer, 1986).

Regarding the chromosome types, the results of present study are coincidently to the previous report (Tanomtong et al., 2011). The autosomes of *C. badius* consisting of 12 large acrocentric, two medium submetacentric, 10 medium acrocentric, four small metacentric, six small submetacentric, eight small acrocentric, six small telocentric and the sex chromosomes are the largest metacentric chromosome.

Interestingly, the active Ag-NOR regions are obviously found in three bi-armed autosomal pairs that are specifically in the telomeric regions of the short arms of those autosome pairs 7, 10 and 17 (Figure 2B). This result may be the first report of NORs detected in the *C. badius* which never reveals before. Theoretically, NORs represent the location of genes that function in ribosome synthesis (Sharma et al., 2002). The most striking variation is found in the morphology of the secondary constrictions. Generally, one major NOR is present per genome (*n*), which may vary in its position between species. However, closely related and often morphologically very similar species share the same type and location of their NORs, which can therefore provide an effective taxonomic marker (King, 1981).

The FISH results using microsatellites $d(CA)_{15}$ probe hybridized on *C. badius* metaphase chromosomes reveal the *in situ* localization positive signals on chromosome pair 13 nearly to subcentromeric regions (Figure 2C). However, microsatellites or simple sequence repeats, generally are located in the heterochromatic regions (telomeres, centromeres and in the sex chromosomes) of genomes. Due to their high polymorphism, co-dominant inheritance, ease of scoring and dense distribution throughout eukaryotic genomes, microsatellites are now generally considered to be the most powerful genetic markers for genetic mapping and evolutionary studies (Supiwong et al., 2014). Here, we suggested that hybridization signal of microsatellite $d(CA)_{15}$ probe was found on chromosome pair 13 and those of three NORs bearing chromosomes can be used as species specific marker for this species. The idiogram from Ag-NOR banding and $d(CA)_{15}$ microsatellite probe is shown in Figure 3.

CONCLUSION

In this study, the results revealed that the karyotype of *C. badius* has the same diploid number $2n=50$ same as previous reports. Here we provide more cytogenetic information that might be used as species specific marker in *C. badius* the NORs bearing chromosome was located on chromosome pairs 7, 10 and 17. The $d(CA)_{15}$ microsatellite also showed hybridization pattern on subcentromeric region in the long arms of chromosomes pair 13. These results provided the knowledge of the chromosomal distribution of repetitive DNA sequences in *C. badius* represents the pioneer for achieving an integrated view of the bamboo rat.

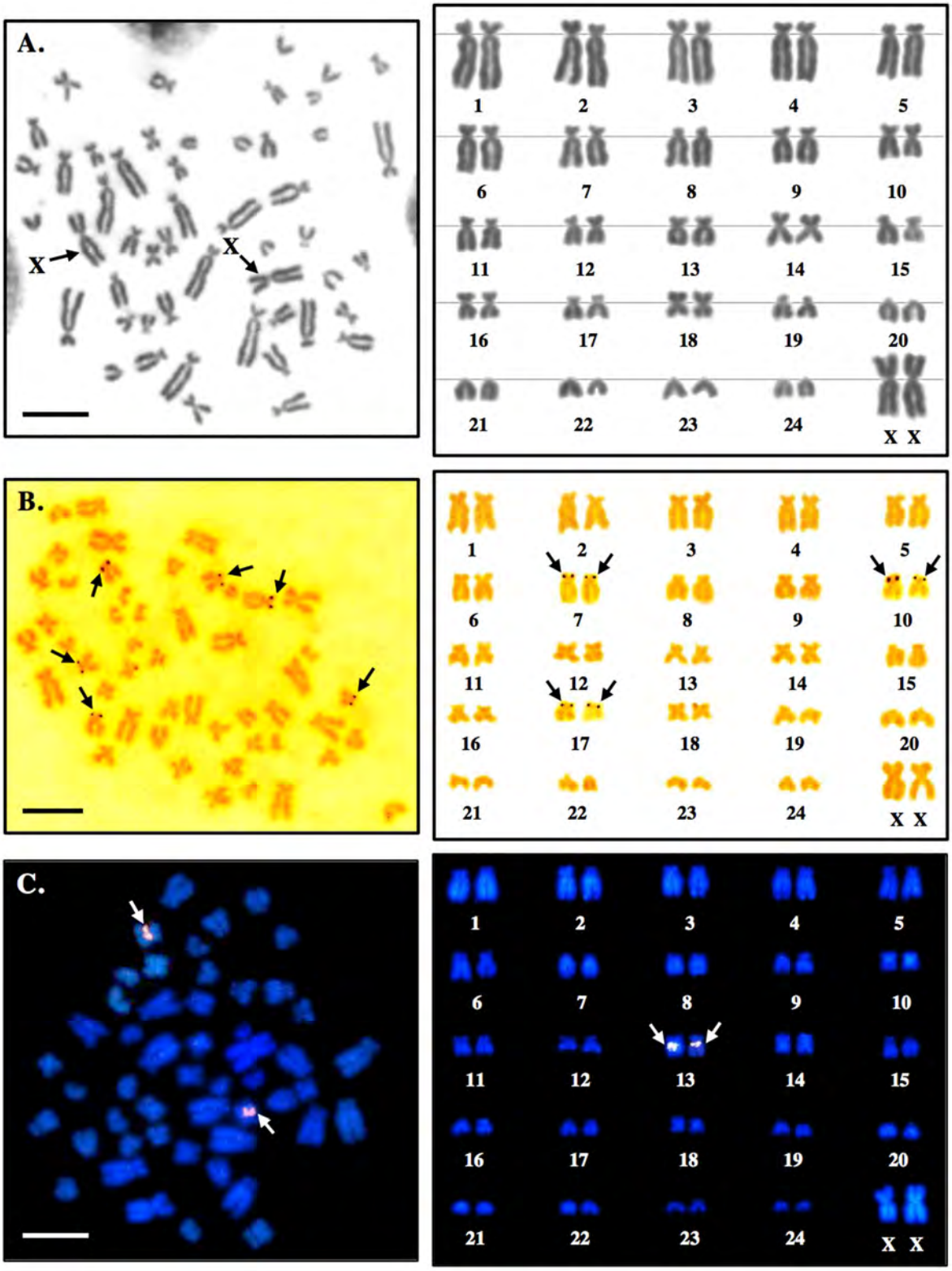

Figure 2. Metaphase chromosome plates and karyotypes of *C. badius* revealed 2*n*=50 by conventional staining (arrows indicate sex chromosomes) [A.], Ag-NOR banding (arrows indicate NORs bearing chromosomes) [B.] and FISH technique using microsatellite d$(CA)_{15}$ probe (arrows indicate positive signal on chromosome pair 13) [C.]. Note scale bars indicate 10 micrometers.

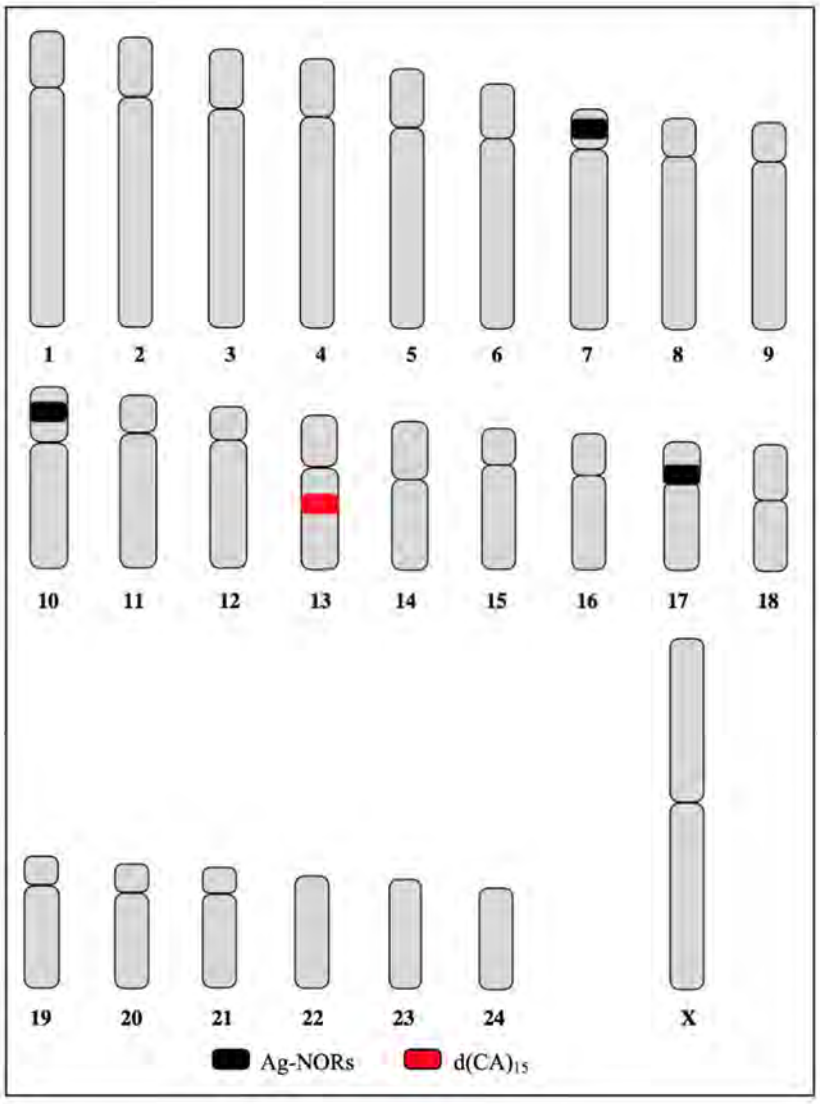

Figure 3. Idiogram showing lengths and shapes of *C. badius*, 2*n*=50 by Ag-NOR banding (black) and d(CA)$_{15}$ microsatellite probe (red).

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