The First Chromosome Analysis and Localization of the Nucleolar Organizer Region of Phayre's Flying Squirrel, *Hylopetes phayrei* (Rodentia, Sciuridae) in Thailand

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Summary We report the first chromosome analysis and localization of the nucleolar organizer region of the Phayre's flying squirrel, *Hylopetes phayrei* (Blyth, 1985) from Thailand. After standard peripheral blood lymphocytes had been cultured at 37°C for 96 h in the presence of colchicine, the metaphase spreads were performed on microscopic slides and air-dried. Conventional staining, GTG-banding, high-resolution and Ag-NOR staining techniques were applied to stain the chromosomes. The results showed that the diploid chromosome number of *H. phayrei* was $2n=38$ and the fundamental number (NF) were 76 in both males and females. The karyotype has the presence of 2 large metacentric, 8 large submetacentric, 8 large acrocentric, 6 medium acrocentric, 6 small metacentric and 6 small acrocentric chromosomes. The X chromosome is a small submetacentric chromosome and the Y chromosome is a small acrocentric chromosome. From the GTG-banding and high-resolution techniques, the numbers of bands and locations in the *H. phayrei* are 182 and 234, respectively; each chromosome pair could be clearly differentiated. The Ag-NOR staining demonstrated that the three pairs of nucleolar organizer regions (NORs) were observed on the long arm regions of chromosome pairs 1, 5, and 14. The karyotype formula of *H. phayrei* could be deduced as:

$$2n \text{ (diploid)} 38 = L_2^m + L_8^{sm} + L_8^a + M_6^a + S_6^m + S_6^a + \text{sex-chromosomes}$$

Key words *Hylopetes phayrei*, Karyotype, Chromosome, Nucleolar organizer region.

Flying squirrels (Petauristinae, Sciuridae, Rodentia) are members of rodents that can make gliding leaps by means of the patagium (parachute-like membrane) connected on each side to their forelegs and hind legs. They are found in North America, Europe and Asia, including at least 37 species grouped into 15 genera. Flying squirrels are special in their life history characteristics. They are nocturnal gliders and hide in holes or leaf drays during the daytime (Nowak 1991). The flying squirrel consists of six genera and 12 species in Thailand, namely, the lesser giant flying squirrel (*Petaurista elegans*), red giant flying squirrel (*Peta. petaurista*), Indian giant flying squirrel (*Peta.*

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Table 1. Review of flying squirrel cytogenetic reports in the subfamily Petauristinae (genera; *Petaurista*, *Pteromys*, *Belomys*, *Hylopetes* and *Glaucomys*).

Species	2n	Karyotype formula	NF	Sex chromosome	NOR-banded	Reference
<i>Pe. petaurista</i>	38	12m+24sm+2a	76	—	—	Nadler and Lay (1971)
<i>Pe. petaurista melanotus</i>	38	22m+14sm	76	X(—), Y(a)	16, 17(TR)	Oshida <i>et al.</i> (1992)
<i>Pe. petaurista grandis</i>	38	22m+14sm	76	X(—), Y(a)	7, 17(TR)	Oshida <i>et al.</i> (1992)
<i>Pe. alborufus castaneus</i>	38	12m+24sm	76	X(sm), Y(a)	8, 13(SCR, TR)	Oshida <i>et al.</i> (2000a)
<i>Pe. alborufus lena</i>	38	22m+14sm	76	X(m), Y(a)	16(TR)	Oshida <i>et al.</i> (2000a)
	38	22m+14sm	76	X(—), Y(a)	16, 17(TR)	Oshida <i>et al.</i> (1992)
<i>Pe. leucogenys</i>	38	22m+14sm	76	X(m), Y(a)	—	Oshida and Obara (1993)
	—	—	—	—	1, 8, 16(TR)	Oshida and Yoshida (1999)
<i>Pe. magnificus</i>	38	18m+16sm+2a	—	X(m), Y(t)	—	Chatterjee and Majhi (1975)
<i>Pe. albiventer</i>	38	—	—	—	—	O'Brien <i>et al.</i> (2006)
<i>Pt. volans</i>	38	12m+22sm+2a	76	X(sm), Y(a)	—	Rausch and Rausch (1982)
	38	10m+22sm+4t	68	X(sm), Y(a)	17(TR)	Oshida <i>et al.</i> (2000b)
	—	—	—	—	17(TR)	Oshida and Yoshida (1999)
<i>Pt. momonga</i>	38	8m+6sm+18a+4t	68	X(sm), Y(a)	5(STR)	Oshida <i>et al.</i> (2000b)
	—	—	—	—	5(STR)	Oshida and Yoshida (1999)
<i>B. pearsonii</i>	38	12m+24sm	76	X(m), Y(a)	1(TR)	Oshida <i>et al.</i> (2002)
<i>H. alboniger alboniger</i>	38	—	76	—	—	Gray and Sharma (1972)
<i>H. phayrei</i>	38	8m+8sm+20a	76	X(sm), Y(a)	1, 5, 14(SCR, STR)	Present study
<i>G. sabrinus laseivus</i>	48	—	74	—	—	Nadler and Sutton (1967)
	48	—	80	—	—	Schindler <i>et al.</i> (1973)
<i>G. volans</i>	48	—	—	—	—	Nadler and Sutton (1967)

Remarks: 2n=diploid chromosome number, NF=fundamental number (number of chromosome arm), m=metacentric, sm=submetacentric, a=acrocentric, t=telocentric, X=X-chromosome, Y=Y-chromosome, STR=subtelocentric region, SCR=subcentromeric region, TR=telomeric region and — = not available.

philippensis), large black flying squirrel (*Aeromys tephromelas*), Phayre's flying squirrel (*Hylopetes phayrei*), Parti-coloured flying squirrel (*H. alboniger*), red-cheeked flying squirrel (*H. spadiceus*), grey-cheeked flying squirrel (*H. lepidus*), white-bellied flying squirrel (*Petinomys setosus*), Vordermann's flying squirrel (*Peti. vordermanni*), hair-footed flying squirrel (*Belomys pearsonii*) and smoky flying squirrel (*Pteromyscus pulverulentus*) (Lekagul and McNeely 1988).

Although phylogenetic studies have been performed paleontologically, morphologically, and molecular biologically, cytogenetic data with banding patterns for these flying squirrel species are still somewhat limited. This paucity of data cannot provide discussion of the karyotypic evolution between flying and non-flying squirrels. However, several studies have appeared dealing with banded karyotypes in several species of the subfamily Petauristinae (Rausch and Rausch 1982, Oshida *et al.* 1992, 2000a, 2000b, 2002, Oshida and Obara 1993, Oshida and Yoshida 1999). There is only one previous report on the genus *Hylopetes*. Gray and Sharma (1972) demonstrated by a conventional staining technique that *H. alboniger alboniger*'s karyotype is 2n (diploid)=38 and the fundamental number (NF) is 76 (Table 1).

In this report, the chromosomal characteristics of *H. phayrei* (Fig. 1) from Thailand are preliminary presented along with the chromosomal localizations of the nucleolar organizer regions (NORs). Several studies have demonstrated variations in the chromosomal localization of NORs, including those of vertebrates using silver staining (Ag-NOR banding technique), which provided useful information of karyotypic evolution (Volleth 1987, Ono and Obara 1994, Mandahl 1979, Winking *et al.* 1980, Shida *et al.* 1999). The number of Ag-NORs was influenced by the activity of rRNA transcription (Babu and Verma 1985, Ono and Yoshida 1998). Also, comparing previous reports of the chromosomes of *H. phayrei* and those of other flying squirrel species, the

Fig. 1. General characteristic of the Phayre's flying squirrel, *Hylopetes phayrei* (Rodentia, Sciuridae) from Thailand.

phylogenetic position of *H. phayrei* within the flying squirrels is only briefly referred to.

Materials and methods

Blood samples from the femoral vein were collected from three males and three females, which were kept in Mahasarakham Province, Thailand using aseptic technique. The samples were kept in 5 mL vacuum tubes containing heparin to prevent blood clotting and were cooled on ice until arriving at the laboratory.

Cell preparation

The lymphocytes were cultured using the peripheral blood microculture technique adapted from Moorhead *et al.* (1960) and Rooney (2001).

Cell culture: The RPMI 1640 medium was prepared with 2% PHA (Phytohemagglutinin) as a mitogen and kept in blood culture bottles of 5 mL each. A blood sample of 0.5 mL was dropped into a medium bottle, mixed, loosely capped, incubated at 37°C under a 5% carbon dioxide environment and regularly shaken in the morning and evening. When reaching harvest time at 72h of incubation, colchicine was introduced and well mixed, followed by further incubation for 30 min.

Cell harvest: The blood sample mixture was centrifuged at 1,200rpm for 10 min and the supernatant was discarded. Ten milliliters of hypotonic solution (0.075 M KCl) was applied to the pellet and the mixture was incubated for 30 min. KCl was discarded with the supernatant after centrifugation again at 1,200rpm for 10 min. Cells were fixed by fresh, cold fixative (methanol: glacial acetic acid=3:1) gradually added up to 8 mL before centrifuging again at 1,200rpm for 10 min, and the supernatant was discarded. The fixation was repeated until the supernatant was clear and the pellet was mixed with 1 mL of fixative. The mixture was dropped onto a clean and cold slide using a micropipette followed by the air-drying technique. The slide was conventionally stained with 20% stock Giemsa's solution for 30 min.

GTG-banding technique

The GTG-banding technique was adapted from Campiranont (2003). The slide was well dried and then soaked in working trypsin (0.025% trypsin EDTA) at 37°C before terminating trypsin activity by washing the slide with Sorensen buffer. The slide was stained with 20% Giemsa's solution for 30 min.

High-resolution technique

The high-resolution technique was adapted from Rooney (2001). After the lymphocytes were cultured for 72h, 0.05 mL of 10^{-5} M methotrexate was applied into the cultured lymphocytes to

Fig. 2. Metaphase chromosome plates and karyotypes of male (A.) and female (B.) Phayre's flying squirrel (*Hylopetes phayrei*) $2n=38$ by conventional staining technique. Arrows indicate satellite chromosomes/NORs (scale bars $10\mu\text{m}$).

induce synchronization. The mixture was incubated again for 17h before the methotrexate was discarded with the supernatant by centrifuging at 2,800rpm. The pellet was mixed with 5 mL of the RPMI 1,640 medium and centrifuged at 2,800rpm. The supernatant was discarded before the cultured cells were released by adding 0.2mL thymidine and incubated for 5h and 15min. The cells were harvested at the exact time and stained by using GTG-banding procedure.

Ag-NOR banding technique

The Ag-NOR banding technique was conducted (Howell and Black 1980) by adding two drops of 50% silver nitrate and 2% gelatin on slides, respectively. The slides were then sealed with cover glasses and incubated at 60°C for 5 min. After that, the slides were soaked in distilled water until the cover glasses were separated. The slide was stained with 20% Giemsa's solution for 1 min.

Chromosomal checks, karyotyping and idiogramming

Chromosome counting was performed on mitotic metaphase cells under a light microscope. Twenty clearly observable and well-spread chromosomes of each male and female were selected and photographed. The length of short arm chromosome (Ls) and the length of long arm chromosome (Ll) were measured and the length of total arm chromosome (LT, $LT=Ls+Ll$) was calculated. The relative length (RL), the centromeric index (CI) and their standard deviation (SD) were estimated (Chaiyasut 1989). The CI ($q/p+q$) between 0.50–0.59, 0.60–0.69, 0.70–0.89 and 0.90–0.99 were described as metacentric, submetacentric, acrocentric and telocentric chromosomes, respectively. The fundamental number (number of chromosome arm, NF) was obtained by assigning a value of two to metacentric, submetacentric and acrocentric chromosomes, and a value of one to telocentric chromosomes. All parameters were used in karyotyping and idiogramming.

Results and discussion

No significant intra-individual variation in chromosome constitution, banding pattern, and chromosome number was observed in any of the specimens examined. The diploid chromosome number ($2n$) was found to be 38 and the fundamental number (NF) 76 in each specimen. Its karyotype was characterized by bi-armed chromosomes. The X-chromosome and Y-chromosome were small among the chromosome complements. The relative lengths of the X-chromosome and Y-chromosome were 0.022 ± 0.001 and 0.016 ± 0.002 , respectively.

Conventional staining karyotype

The cytogenetic study of *H. phayrei* using lymphocyte culture and the conventional staining procedures revealed that the chromosome number was $2n$ (diploid)=38, which consists of 36 (18 pairs) autosomes and two (1 pair) sex-chromosomes. The NF of *H. phayrei* was 76 in both males and females. According to Gray and Sharma (1972), who reported the chromosome number and NF of *H. alboniger alboniger* to be 38 and 76, respectively, the chromosome number and NF of flying squirrel species in the genus *Hylopetes* are all the same, and our results agree. The karyotype of *H. phayrei* was composed of 2 large metacentric, 8 large submetacentric, 8 large acrocentric, 6 medium acrocentric, 6 small metacentric and 6 small acrocentric chromosomes (Fig. 2). Cytogenetic studies on the flying squirrel are few. Fourteen species have been studied cytogenetically, each having the same diploid number ($2n=38$) (Nadler and Lay 1971, Gray and Sharma 1972, Chatterjee and Majhi 1975, Oshida *et al.* 1992, 2000a, 2000b, 2002, Oshida and Obara 1993, Rausch and Rausch 1982, Oshida and Yoshida 1999, O'Brien *et al.* 2006). In some species, a different diploid number from these studies has been reported, such as $2n=48$ for *Glaucomys sabrinus laseivus* and *G. volans* (Nadler and Sutton 1967, Schindler *et al.* 1973).

Ag-NOR banded karyotype

Our cytogenetic study of *H. phayrei* performed by Ag-NOR staining technique is the first of its kind. The objective of this technique is to detect the nucleolar organizer regions (NORs) (satellite chromosome), which represent the location of genes that function in ribosome synthesis (18S and 28S ribosomal RNA). NORs produce numerous gene expressions, and they are composed of more non-histone protein than other chromosome regions. These proteins induce specific dark bands by the reduction of organic silver that changes silver to be dark (Gold *et al.* 1990). In *H. phayrei*, Ag-NOR bands were observed on the satellite stalk and the subtelomeric regions of the long arm of the chromosome pairs 1 and 5 (four positions), and the subcentromeric regions of the long arm of the chromosome pair 14 (two positions) (Fig. 3).

Twelve species of flying squirrels have been investigated by the Ag-NOR staining technique, some of which have been applied to elucidate taxonomic and morphological affinities, *e.g.* in the

Fig. 3. Metaphase chromosome plates and karyotypes of female (A.) and male (B.) Phayre's flying squirrel (*Hylopetes phayrei*) $2n=38$ by Ag-NOR banding technique. Arrows indicate nucleolar organizer regions/NORs (scale bars $10\mu\text{m}$).

genus *Petaurista* (Oshida *et al.* 1992, 2000a, Oshida and Yoshida 1999). Moreover, the amount and location of NORs can explain the evolution of each chromosome. Normally, most flying squirrels (genera *Petaurista*, *Pteromys* and *Belomys*) have one to two pairs of small NORs in a chromosome complement. If some flying squirrel (genus *Hylopetes*) has more than four NORs, it may have been caused by a translocation between a NOR containing chromosome and another chromosome. Furthermore, NORs are usually located close to a telomere of a chromosome arm. If a NOR appears between a centromere and a telomere, it may be caused by the centric fusion or pericentric inversion between two telocentric chromosomes.

GTG-banded and high-resolution karyotype

The GTG-banding and high-resolution techniques provide a clear chromosome band, which are represented by positive-black regions (heterochromatin) and negative-white regions (euchromatin) on autosomes and sex-chromosomes. The GTG-banding revealed that the number of

Fig. 4. Metaphase chromosome plates and karyotypes of male (A.) and female (B.) Phayre's flying squirrel (*Hylopetes phayrei*) $2n=38$ by GTG-banding technique. Arrows indicate nucleolar organizer regions/NORs (scale bars $10\mu\text{m}$).

G-bands on one set of haploid chromosomes (n), which includes autosomes and the X and Y chromosomes is 182 bands for the *H. phayrei*. The number of bands in one set of prometaphase haploid chromosomes from the high-resolution technique is 234 bands (Figs. 4 and 5). Stanyon (1987) suggested that the high-resolution technique provides high efficiency for chromosome difference comparison. The evolution study of gorillas, orangutans, chimpanzees and humans by the comparison of chromosome structure and chromosome bands with GTG-banding and high-resolution techniques gave chromosome band numbers of 320–500 and over 1,000 bands per haploid set (Yunis and Prakash 1982). In this study, the chromosome scoring is done with only clearly visible bands except for the variable band due to the small number of scored bands.

The high-resolution technique provides the possibility to detect chromosome breaks and rearrangement events within major bands. The developments in cell culture and banding techniques have been extremely fast; 10 years after the Paris Conference in 1971, Yunis (1981) published the haploid human karyotype at the 1,700-band stage. For other mammals, the development was slower, but recently, good results have narrowed the gap between human and animal cytogenetics. However, the increase of bands in a given karyotype is not an aim by itself, but a research tool, so

Fig. 5. Prometaphase chromosome plates and karyotypes of male (A.) and female (B.) Phayre's flying squirrel (*Hylopetes phayrei*) $2n=38$ by high-resolution technique. Arrows indicate nucleolar organizer regions/NORs (scale bars $10\mu\text{m}$).

the value of a high band level is reduced if band quality is sacrificed in the process. When high-resolution banding is combined with other chromosomal techniques, specific sites on the chromosomes can be detected and precisely localized. The high-resolution technique has been extremely valuable, especially for the localization of single copy genes and specific breakpoints (Rønne 1991).

Chromosome markers

For *H. phayrei*, chromosome markers are the autosome pair 1 and pair 21, which are the largest submetacentric chromosome and the smallest metacentric chromosome, respectively. The important karyotype feature of *H. phayrei* is the asymmetrical karyotypes, which were found in all three types of chromosomes (metacentric, submetacentric and acrocentric chromosomes). The largest chromosome is four times larger than the smallest chromosomes. Figures 6–9 show the idiogram of the *H. phayrei* from conventional staining, Ag-NOR banding, GTG-banding and high-

Fig. 6. Idiogram of Phayre's flying squirrel (*Hylopetes phayrei*) $2n=38$ by conventional staining. Arrows indicate nucleolar organizer regions (NORs).

Fig. 7. Idiogram of Phayre's flying squirrel (*Hylopetes phayrei*) $2n=38$ by Ag-NOR banding technique. Arrows indicate nucleolar organizer regions (NORs).

Fig. 8. Idiogram of Phayre's flying squirrel (*Hylopetes phayrei*) $2n=38$ by GTG-banding technique. Arrows indicate nucleolar organizer regions (NORs).

Fig. 9. Idiogram of Phayre's flying squirrel (*Hylopetes phayrei*) $2n=38$ by high-resolution technique. Arrows indicate nucleolar organizer regions (NORs).

Table 2. Mean lengths of short arm chromosome (Ls), long arm chromosome (LI), and total arm chromosome (LT), relative length (RL), centromeric index (CI), and standard deviation of RL and CI from 20 metaphases of male and female Phayre's flying squirrel (*Hylopetes phayrei*), $2n=38$.

Chromosome pair	Ls	LI	LT	CI±SD	RL±SD	Chromosome size	Chromosome type
1*	74.340	113.499	187.838	0.604±0.020	0.045±0.002	Large	Submetacentric
2	32.549	115.782	148.331	0.779±0.123	0.035±0.004	Large	Acrocentric
3	57.174	87.293	144.467	0.603±0.026	0.034±0.002	Large	Submetacentric
4	33.955	108.557	142.511	0.760±0.105	0.034±0.002	Large	Acrocentric
5*	42.971	96.328	139.299	0.692±0.033	0.033±0.002	Large	Submetacentric
6	31.836	96.232	128.067	0.751±0.061	0.030±0.002	Large	Acrocentric
7	58.838	64.816	123.653	0.524±0.018	0.029±0.002	Large	Metacentric
8	29.127	93.837	122.963	0.758±0.081	0.029±0.001	Large	Acrocentric
9	40.217	82.351	122.568	0.675±0.062	0.029±0.001	Large	Submetacentric
10	25.566	89.413	114.942	0.776±0.058	0.027±0.003	Medium	Acrocentric
11	28.033	85.999	113.541	0.763±0.137	0.027±0.003	Medium	Acrocentric
12	28.449	71.152	99.143	0.720±0.062	0.024±0.001	Medium	Acrocentric
13	23.842	69.809	93.518	0.745±0.035	0.022±0.002	Small	Acrocentric
14*	23.266	64.281	86.979	0.741±0.070	0.021±0.002	Small	Acrocentric
15	37.648	42.939	80.187	0.537±0.065	0.019±0.001	Small	Metacentric
16	19.454	54.370	73.209	0.745±0.047	0.017±0.003	Small	Acrocentric
17	26.366	30.059	56.345	0.534±0.032	0.013±0.001	Small	Metacentric
18	20.918	23.225	44.143	0.527±0.030	0.011±0.002	Small	Metacentric
X	34.755	57.828	92.583	0.627±0.032	0.022±0.001	Small	Submetacentric
Y	17.822	50.651	68.473	0.738±0.027	0.016±0.002	Small	Acrocentric

Remark: * = NOR-bearing chromosomes (satellite chromosomes).

resolution techniques, respectively. The idiogram shows a gradually decreasing length of the chromosomes. The chromosome length in centimeters of 20 cells (males and females) in mitotic metaphase was measured. The mean length of short arm chromosome (Ls), length of long arm chromosome (LI), total length of arm chromosome (LT), relative length (RL), centromeric index (CI), standard deviation of RL and CI, and the size and type of chromosomes are shown in Table 2. The karyotype formula for *H. phayrei* could be deduced as:

$$2n \text{ (diploid)} 38 = L_2^m + L_8^{sm} + L_8^a + M_6^a + S_6^m + S_6^a + \text{sex-chromosomes}$$

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