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Classical chromosome features and microsatellites repeat in *Gekko petricolus* (Reptilia, Gekkonidae) from Thailand

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Abstract. The objectives of this study were to examine size, shape, diploid number (2n), fundamental number (NF), NORs region, and distribution of microsatellite by using Fluorescence *in situ* hybridization technique (FISH) and to establish the karyotype and standard idiogram of sandstone geckos (*Gekko petricolus* Taylor, 1962). Sandstone gecko distributed in the sandstone mountains in Laos, Cambodia, and Thailand. Five male and five female specimens were collected from Ubon Ratchathani and Mukdahan provinces, Thailand. The metaphase cells were directly prepared from the bone marrow cells. Chromosomes were stained by conventional staining, NORs-banding and FISH techniques. The results found that the diploid number was 38 chromosomes. The fundamental number was 54. The karyotype composed of 4 large metacentric, 4 large acrocentric, 2 large telocentric, 4 medium acrocentric, 2 medium telocentric, 2 small submetacentric, 2 small acrocentric and 18 small telocentric chromosomes. No morphological difference was identified between sex chromosomes of male and female specimens. The NORs appeared to telomere of the long arm of chromosome pair 17. The study displayed that the distribution of microsatellite using $(CA)_{15}$ and $(GAA)_{10}$ probes distributed throughout the genome. However, $(CA)_{15}$ sequences concentrated in the telomere. The karyotype formula *G. petricolus* is as follow: $2n$ (38) = $L^m_4+L^a_4+L^t_2+M^a_4+M^t_2+S^{sm}_2+S^a_2+S^t_{18}$.

Keywords: Karyotype, *Gekko petricolus*, Ag-NOR, FISH microsatellite.

INTRODUCTION

The sandstone gecko (*Gekko petricolus*) belongs to family Gekkonidae, a genus mainly found in subtropical limestone areas, near the Tropic of Cancer, such as southern China, India, Myanmar, Thailand, Vietnam, Malaysia, Indonesia, and other countries in Southeast Asia (Li et al. 1996).

The genus *Gekko* comprises over 30 species and a few undescribed species distributed in East and Southeast Asia, and western Oceania (Kluge 2001; Toda 2008). The *Gekko petricolus* group currently contains 10 species, namely *G. boehmei*, *G. badenii*, *G. canaensis*, *G. flavimaritus*, *G. grossmanni*, *G. lauhachindai*, *G. petricolus*, *G. russelltraini*, *G. takouensis*, and *G. vietnamensis*. (Ngo et al. 2009; Panitvong et al. 2010; Ngo and Gamble 2011; Rösler et al. 2011; Luu et al. 2015; Rujirawan et al. 2019). Karyological analyses in *Gekko* have differentiated species based on mitotic metaphase chromosomal morphology while sporadic reports have based the species differentiation on meiotic metaphase chromosomal morphology. The chromosome study of Gekkonid that have been reported such as; *Hemidactylus*: diploid number (2n) ranging from 40-56 and mostly 40 or 46 (De Smet 1981; Patawang and Tanomtong 2015b), *Gehyra*: mostly 44 (King, 1984), *Ptychozoon*: 2*n* = 34 and 42 (Ota and Hikida, 1988), *Paroedura*: diploid number ranging from 31-38 and mostly 36 (Aprea et al. 2013; Koubova et al. 2014), *Phelsuma*: 2*n* = 36 (Aprea et al., 1996), *Dixonius*: 2*n* = 42 (Ota et al., 2001), and genus *Gekko,* there are several reports on cytogenetic studies of *G. gecko*, namely, *G. gecko*: 2n=38 (Singh 1974; Wu and Zhao 1984; Solleder and Schmid, 1984; Trifonov et al. 2011; Qin et al. 2012; Patawang et al. 2014), *G. hokouensis*: 2n=38 (Chen et al. 1986; Shibaike et al. 2009; Kawai et al. 2009), *G. japonicus*: 2n=38 (Yoshida and Itoh, 1974; Shibaike et al., 2009; Trifonov et al., 2011), *G. shibatai* and *G. vertebralis*: 2n=38 (Shibaike et al. 2009), *G. Vittatus* and *G. ulikovskii*: 2n=38 (Trifonov et al. 2011), *G. tawaensis*: 2n=38 (Ota, 1989a; Shibaike et al. 2009), *G. taylori*: 2n=42 (Ota and Nabhitabhata 1991), *G. monarchus*: 2n=44 (Ota et al. 1990), *G. yakuensis*, *G. petricolus* and *G. smithii*: 2n=38-42 (Ota, 1989), *G. kikuchii*: 2n=44 (Ota, 1989a), *G. chinensis*: 2n=40 (Lau et al. 1997), *G. subpalmatus*: 2n=38 (Wu and Zhao 1984), *G. swinhonis*: 2n=38 (Chen et al. 1986) (Table 1). Most gekkonid chromosome complements consist of acrocentric or telocentric chromosomes which gradually decreases in size whereas the karyotype evolution within the group is accompanied by fusions, Robertsonian fissions and pericentric inversions (Gorman 1973). From the previous reports, there are no studies of *G. petricolus* chromosome or karyotypic analyses. The present study of the cytogenetics of *G. gecko* provides the first report on the conventional staining, Ag-NOR banding and fluorescence *in situ* hybridization techniques. Data provided here can gain us the knowledge of cytogenetic information which can be used as a basis to comprehensively examine the taxonomy and evolutionary relationship of *Gekko* species.

MATHERIAL AND METHODS

Sample collection

We obtained five male and five female specimens of *G. petricolus* (Fig. 1) that were collected from Ubon Ratchathani and Mukdahan provinces, Northeastern Thailand.

Chromosome preparation

Chromosomes were directly prepared *in vivo* (Ota 1989a; Qin et al. 2012) using the following methods. The gecko intramuscular was inoculated Colchicine solution then left for 12 h. After that cut testis samples and bone marrow into small pieces. Then squashed and mixed with 0.075 M KCl. After discarding all large piece tissues, 15 mL of cell sediments were transferred to a centrifuge tube and incubated for 25–35 min. The KCl was discarded from the supernatant after centrifugation again at 3,000 rpm for 8 min. In fresh cool fixative, cells were fixed (3 methanol : 1 glacial acetic acid), gradually added to make a volume of 8 mL, before being centrifuged again at 3,000 rpm for 8 min. Afterward the supernatant was expelled. Fixation was repeated until the supernatant was clear whereas the pellet was mixed with 1 mL fixative. The mixture was dropped onto a clean and cold slide by micropipette followed by air-drying technique.

Chromosome staining

With 20% of Giemsa solution, the slides were conventionally stained for 30 minutes (Patawang et al. 2014). After that, to remove excess stain, the slides were rinsed thoroughly with running tap water and were placed in air-dry at room temperature. Ag-NOR banding was analysed according to the method of Howell and Black (1980). Two drops each of 50% silver nitrate and 2% gelatine solutions were added to slides, respectively. Then, they were sealed with cover glasses and incubated at 60°C for 5-10 minutes. They were also soaked in distilled water until the cover glasses were separated. Finally, the slides were placed in air-dry at room temperature. They were observed under microscope.

Chromosome checks

Metaphase figures were analysed following the chromosome classification of Turpin and Lejeune (1965). Chromosomes were categorized as submetacentric (sm),

Table 1 The karyotype reviews among the genes *Gekko* (Gekkonidae, Squamata).

Species	2*n*	NF	Karyotype formulas	NORs	FISH	Locations	References
Gekko gekko	38	50	6m+4sm+2a+26t	P4	-	Thailand	Patawang (2014)
	38	44	6bi-arms+32uni-arms	-	-	-	Singh (1974)
	38	-	-	-	-	-	Wu and Zhao (1984)
	38	46	8bi-arms+30uni-arms	-	-	-	Solleder and Schmid (1984)
	38	-	-	-	-	-	Trifonov *et al.* (2011)
	38	50	8 m+2sm+2st(a)+26t			Laos	Qin *et al.* (2012)
	38	48	8 m+2sm+28t	-	-	China	Qin *et al.* (2012)
G. hokouensis	38	56	4 m+6sm+20t+8bi-arms*	P19	-	China,	Chen *et al.* (1986)
	38	58/59	2 m+8sm+10a+16t+Z(t)+W(a)	-	-	Japan	Kawai *et al.* (2009)
	38	58	4 m+8sm+18t+8bi-arms*	P19	-	Taiwan	Shibaike *et al.* (2009)
	38	58/59	4 m+8sm+16t+8bi-arms*+Z(t)W(sm)	P19	-	Japan	Shibaike *et al.* (2009)
	38	42	2m+18sm+16a+ZW	P19	FISH mapping	Thailand	Srikulnath (2015)
G. japonicus	38	-	-	-	-	-	Yoshida and Itoh (1974)
	38	58	4m+8sm+8st+18t	P17	-	Japan	Shibaike et al. (2010)
	38	-	-	-	-	-	Trifonov *et al.* (2011)
G. shibatai	38	58	4m+8sm+18t+8bi-armed	P19	-	Japan	Shibaike et al. (2009)
G. vertebralis	38	62	4m+14sm+14t+6bi-arme*	P19	-	Japan	Shibaike et al. (2009)
G. vittatus	38	-	-			-	Trifonov *et al.* (2011)
G. ulikovskii	38	-	-			-	Trifonov *et al.* (2011)
G. tawaensis	38	58	4m+8sm+18t+8bi-armed	P19	-	Japan	Shibaike et al. (2009)
	38	56	-	P19	-	Japan	Ota (1989a)
G. taylori	42	-	-	-	-	Thailand	Ota and Nabhitabhata (1991)
G. monarchus	44	46	-	-	-	Malaysia	Ota et al. (1990)
G. yakuensis	38	56	-	P19	-	Japan	Ota (1989a)
G. petricolus	38	54	-	-	-	Thailand	Ota (1989a)
G. kikuchii	44	50	-	-	-	Taiwan	Ota (1989a)
G. chinensis	40	46	6bi-armed+34uni-armed	-	-	China	Lau et al. (1997)
G. smithii	38	48	-	-	-	-	Ota (1989a)
G. subpalmatus	38	58	-	-	-	-	Wu and Zhao (1984)
G. swinhonis	38	66	-	-	-		Chen *et al.* (1986)
G. petricolus	38	54	4m+2sm+10a+22t	P17	$(CA)_{15}$, $(GAA)_{10}$	Thailand	Present study

Remarks: 2n = diploid chromosome number, NORs = nucleolar organizer regions, NF = fundamental number (number of chromosome arms), bi-arm = bi-armed chromosome, m = metacentric, sm = submetacentric, a = acrocentric, t = telocentric chromosome, *=small bi-arms chromosome, and - = not available.

Figure 1. General characteristic of *G. petricolus* (scale bar = 5 cm).

metacentric (m), telocentric (t), and acrocentric (a). The Fundamental Number (NF: number of chromosome arms) was gained by assigning a value of two to metacentric, submetacentric and acrocentric chromosomes and one to acrocentric chromosomes.

Fluorescence in situ hybridization technique

The use of microsatellite investigations which were described by Kubat et al. (2008) was followed here with slight modifications. These sequences 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 rigorous conditions on mitotic chromosome spreads (Pinkel et al. 1986). After denaturation of chromosomal DNA in 70% formamide/ 2×SSC at 70 °C, spreads were incubated in 2×SSC for 4 min at 70 °C. The hybridization mixture (2.5 ng/μL probes, 2 μg/μL salmon sperm DNA, 50% deionized formamide, 10% dextran sulphate) 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 fulfilled with 1×SSC for 5 min at 65 °c. A final wash was operated at room temperature in 4×SSCT for 5 min. 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.

RESULTS AND DISCUSSION

Mitotic chromosome features from Giemsa staining

Karyomorphology of the *G. petricolus* revealed that the number of diploid chromosome (2*n*) was 38 and the fundamental number was 54. The karyotype composed of 4 large metacentric, 4 large acrocentric, 2 large telocentric, 4 medium acrocentric, 2 medium telocentric, 2 small submetacentric, 2 small acrocentric and 18 small telocentric chromosomes. (Table 2 and Figs. 1A-B). The karyotype formula of *G. petricolus* 2*n* (38) = $L^m_4+L^a_4+L^t_2+M^a_4+M^t_2+S^{sm}_2+S^a_2+S^t_{18}$. The diploid chromosome number is following previous studies of *Gekkos*. However, overall karyotypes of *G. petricolus was* similar to other *Gekko*, diploid number ranging from 38-42 and mostly 38 (Singh 1974; Wu and Zhao 1984; Solleder and Schmid 1984; Yoshida and Itoh 1974; Ota 1989a; Ota et al. 1990; Ota and Nabhitabhata 1991; Lau et al. 1997; Chen et al. 1986; Kawai et al. 2009; Shibaike et al. 2009; Trifonov et al. 2011; Qin et al. 2012; Patawang et al. 2014). Proximity of chromosome number and karyotype feature within genus *Gecko* represent a close evolutionary line in the group. This species exhibit no sex differences in karyotypes between males and females (Figs. 2A-B). No cytologically distinguishable sex chromosome was observed to be similar to *G. shibatai*, *G. tawaensis*, *G. yakuensis*, *G. vertebralis*, *G. japonicas*, *G. vittatus*, *G. ulikovskii*, *G. chinensis*, *G. kikuchii*, *G. monarchus*, *G. petricola*, *G. smithii*, *G. subpalmatus*, *G. swinhonis* and *G. Taylori* (Shibaike et al. 2009) and other lizards in Thailand (Satrawaha and Ponkanid, 1988, Wongwattana et al., 2001). This study is different from the study of Kawai

Table 2. Karyomorphological details of the *G. petricolus,* 2n = 38.

Chro. Pair	Ls (μm)	Ll (μm)	LT (μm)	RL±SD	CI±SD	Sizes	Types
1	2.350	3.284	5.563	0.116±0.006	0.589±0.017	Large	metacentric
2	2.237	2.906	5.143	0.107±0.005	0.556±0.024	Large	metacentric
3	0.967	3.130	4.047	0.085±0.003	0.767±0.033	Large	acrocentric
4	0.833	2.986	3.790	0.079±0.003	0.781±0.029	Large	acrocentric
5	0.000	3.294	3.229	0.067±0.004	1.000±0.000	Large	telocentric
6	0.000	3.085	3.050	0.063±0.002	1.000±0.000	Medium	telocentric
7	0.656	2.354	2.953	0.062±0.002	0.792±0.025	Medium	acrocentric
8	0.536	2.269	2.787	0.058±0.003	0.813±0.034	Medium	acrocentric
9	0.000	2.442	2.420	0.050±0.002	1.000±0.000	Small	telocentric
10	0.000	2.272	2.236	0.047±0.002	1.000±0.000	Small	telocentric
11	0.000	1.972	1.941	0.041±0.003	1.000±0.000	Small	telocentric
12	0.000	1.817	1.774	0.037±0.002	1.000±0.000	Small	telocentric
13	0.000	1.696	1.653	0.034±0.002	1.000±0.000	Small	telocentric
14	0.000	1.544	1.515	0.031±0.002	1.000±0.000	Small	telocentric
15	0.000	1.455	1.415	0.029±0.002	1.000±0.000	Small	telocentric
16	0.374	1.049	1.388	0.029±0.002	0.748±0.039	Small	acrocentric
17*	0.466	0.831	1.266	0.027±0.002	0.656±0.059	Small	submetacentric
18	0.000	0.971	0.969	0.020±0.002	1.000±0.000	Small	telocentric
19	0.000	0.838	0.857	0.018±0.002	1.000±0.000	Small	telocentric

Remarks: Ls=Short arm, Ll=Long arm, LT=Total chromosome length, CI=Centromeric Index, RL=Relative length, *=NORs bearing chromosomes (satellite chromosome)

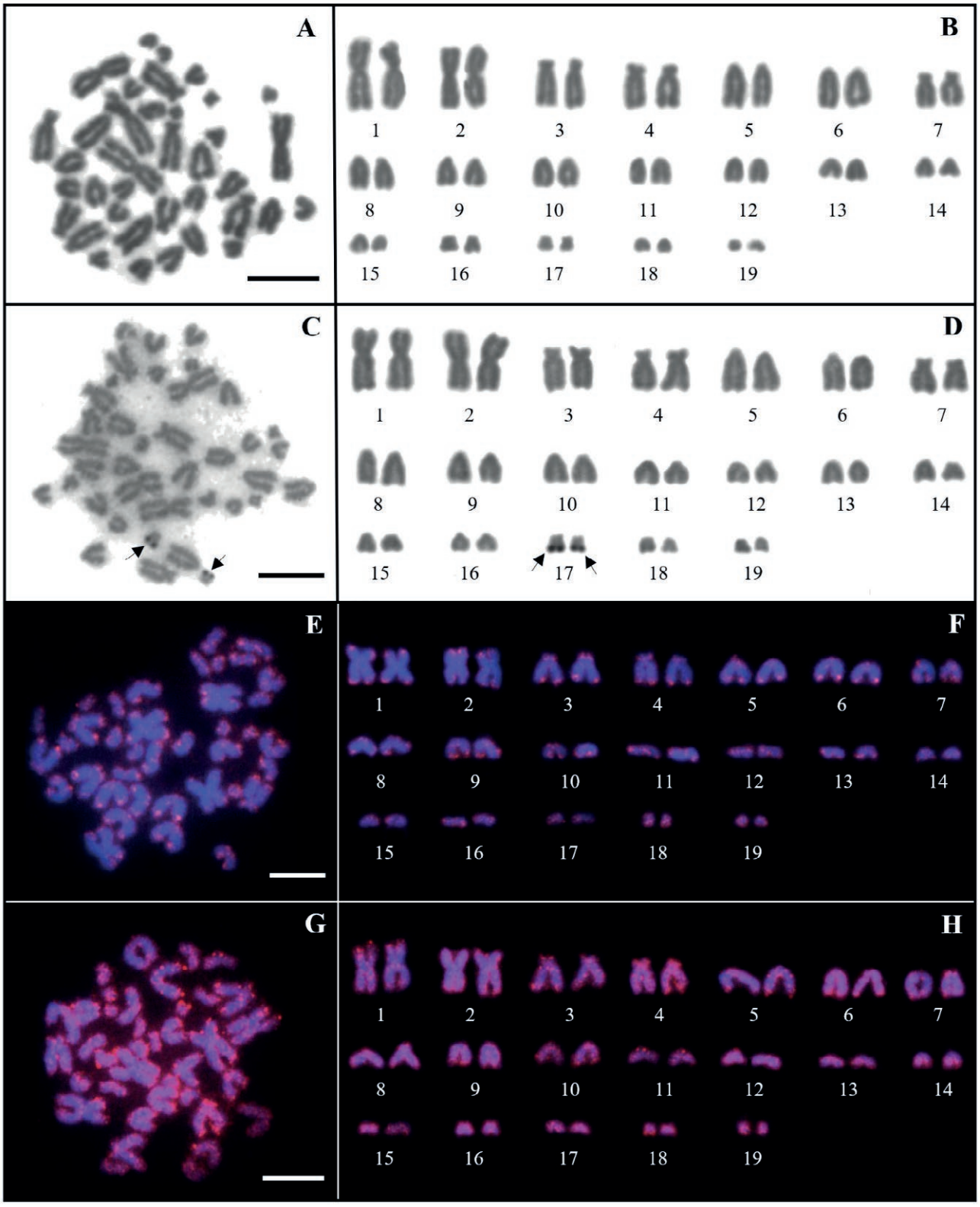

Figure 2. Metaphase chromosome plate and karyotypes of *G. petricolus* (**A, B**) by conventional technique, (**C, D**) by Ag-NOR banding technique (**A, B**) by FISH technique of microsatellite probe $(CA)_{15}$, (**G, H**) by microsatellite probe $(GAA)_{10}$. Scale Bars = 10 μm.

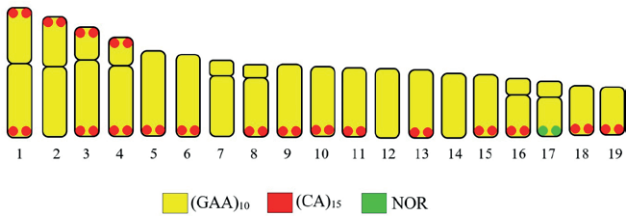

Figure 3. Idiogram represents the microsatellite probes $(CAA)_{10}$, $(CA)_{15}$ and Ag-NOR banding on the chromosomes of *G. petricolus*.

et al. (2009) and Shibaike et al. (2009) which revealed a ZW system (sex-chromosomes) of *G. hokouensis* in Japan. Geckos represent an interesting group regarding the evolution of sex determination mechanisms and include species possessing either environmental or genetic sex determination systems. Gecko populations without males were first discovered followed by seven parthenogenetic species (Smith 1935; Ota 1989b; Volobouev et al. 1993), including triploid (3*n*) forms in some populations (Moritz 1984). A ZW system was recently revealed in *G. hokouensis*, and the genetic content of its sex chromosomes was similar to that of the avian Z chromosome (Kawai et al. 2009; Shibaike et al. 2009).

Nucleolar organizer region

The first cytogenetic study of *G. petricolus* carried out by Ag-NOR banding technique was obtained from this research. We found the observable NORs on the region adjacent to telomere of long arm of the submetacentric chromosome pair 17th (Figs 2C-D). The objective of the Ag-NOR banding technique is to detect NORs which represent the location of genes that have a 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). This study according to *G. japonicas* had two NORs on the long arm near telomere of small bi-arms chromosome pair 17 (Shibaike et al. 2009). The NOR regions compared with other geckos, most showed two NORs appearing near telomeric region of small bi-armed or small mono-armed chromosome. An example of the previous reports of the genus *Gekko* had two NORs on one pair of small bi-arms chromosomes. *G. gecko* had two NORs on the near telomere of mono-arms chromosome pair 4 (Patawang 2014), while there were *G. shibatai*, *G. Yakuensis, G. hokouensis*, *G. tawaensis*, *G. vertebralis* and *G. yakuensis* which had two NORs on the long arm near telomere of small bi-arms chromosome pair 19 (Ota 1989b; Chen et al. 1986; Shibaike et al. 2009). The use of NORs in explaining kinships depends a large extent on the uniformity of this characteristic and the degree of variety within a taxon (Yüksel and Gaffaroğlu 2008).

Microsatellite pattern

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 and Renz 1984; Ellegren 2004; Chistiakov et al. 2006). They show ample distribution throughout eukaryotic genomes, being scattered or clustered both in euchromatin and heterochromatin. They are highly polymorphic regarding copy number deviation (Ellegren 2004). Fluorescence hybridization indicate the $(CA)_{15}$ repeat showing abundance at the telomeric ends of most chromosomes (Figs. 2E-F), verifying the findings from other gekko groups investigated to date (Srikulnath 2015). Hybridization signals of $(GAA)_{10}$ repeats were studied at all chromosomes (Figs. 2G-H), while the results clearly showed that the microsatellite repeats are in high copy number on some chromosome pairs, according to previous reports on reptile groups (Pokorná et al. 2011; Matsubara et al., 2013). In this study, a comparison of the cytogenetic maps of *G. hokouensis*, enabled us to describe the processes of chromosomal reorganization in Gekkota. These cytogenetic data could also be a substantial prerequisite the reptiles' genome projects in the future.

CONCLUSIONS

This study, The first cytogenetic study of *G. petricolus*. Karyotyping from metaphase spreads of *G. petricolus* showed a chromosome number of 2n = 38, NF=54. The karyotype formula is $2n\ (38) = L^{m}_{4}+L^{a}_{4}+L^{t}_{2}+M^{a}_{4}+M^{t}_{2}+S^{sm}_{2}+S^{a}_{2}+S^{t}_{18}$. Data obtained in this study can increase the knowledge of cytogenetic which can be used as a basis to comprehensively examine the taxonomy and evolutionary relationship of *gekko* species and other gekkonid.

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