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First Report on Classical and Molecular Cytogenetics of Doi Inthanon Bent-toed Gecko, *Cyrtodactylus inthanon* Kunya *et al.*, 2015 (Squamata: Gekkonidae) in Thailand

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Abstract. This study analyzed the karyotype of *Cyrtodactylus inthanon* Kunya *et al.*, 2015 from Doi Inthanon, Chiang Mai Province, northern Thailand. The metaphase and meiotic chromosome preparations were obtained by squash technique from bone marrow and testes, respectively. The chromosomes were stained by Giemsa staining, Ag-NOR banding and molecular cytogenetics with fluorescence in situ hybridization (FISH) using microsatellites $d(CA)_{15}$, $d(GC)_{15}$, $d(CAG)_{10}$ and $d(GAA)_{10}$ as probes. The results showed the diploid chromosome number ($2n$) of 40. The chromosome types of metacentric, submetacentric, acrocentric and telocentric chromosomes were 12-4-2-22, respectively. The Ag-NORs banding technique provides the pair of nucleolar organizer regions (NORs) on the telomeric region of the long arm of acrocentric pair 12. There are no sex differences in karyotypes between males and females. We also found that during metaphase I on meiosis of *C. inthanon*, the homologous chromosomes appeared synapsis of 20 bivalents. The microsatellite $d(CA)_{15}$ signals were located on the sub-centromeric region of the metacentric pair 10, whereas the $d(GC)_{15}$, $d(CAG)_{10}$ and $d(GAA)_{10}$ repeats are highly accumulated throughout almost all entire chromosomes. The karyotype formula is as follows: *C. inthanon* ($2n$ = 40), $L^m_2 + L^{sm}_4 + L^t_6 + M^m_2 + M^t_8 + S^m_8 + S^a_2 + S^t_8$.

Keywords: Bent-toed Gecko, *Cyrtodactylus inthanon*, Ag-NOR staining, karyotype, FISH.

INTRODUCTION

Cyrtodactylus is a genus of bent-toed geckos which is widely distributed across South Asia to Melanesia (Wood *et al*. 2012, Grismer *et al*. 2020, 2021). This genus is the most species group of gekkotans, with approximately 306 species currently recognized (Uetz *et al*. 2021). The Gekkonidae found in Thailand are highly diverse and include approximately 90 species. Among these species, 38 species of bent-toed geckos (*Cyrtodactylus*) are the most well-documented and show wide distribution in Thailand (Uetz *et al*. 2021). Although a high number of *Cyrtodactylus* species have already been discovered and many more explorations are anticipated based on the rapid discovery rate of new *Cyrtodactylus* species, several are defined as endemic including *C. sanook*, *C. saiyok* and *C. phuketensis* (Panitvong *et al*. 2014, Pauwels *et al*. 2013, Sumontha *et al*. 2012). Recently, *C. Inthanon*, Doi Inthanon bent-toed gecko was discovered as a new species from Doi Inthanon region, Chiang Mai Province, northern Thailand by Kunya *et al*. (2015). It was a novel reptile endemic to Doi Inthanon strengthening the high significance of this mountain in terms of biodiversity preservation. In terms of endemic species, they are at higher risk of extinction because their habitat is limited or unique. Additionally, they have been reported in recent years that the rate of destruction of *Cyrtodactylus* habitat has increased due to both forest land encroachment and wildfires, especially in Southeast Asia where they are mainly distributed (Chomdej *et al*. 2021).

However, cytogenetic studies are still quite scarce within *Cyrtodactylus*, mostly restricted to classical protocols. Studies applying molecular cytogenetic approaches (i.e. chromosomal mapping of microsatellite sequences) where done only in two species of *C. jarujini* and *C. doisuthep* (Thongnetr *et al*. 2021). Up to date only seven species from over 306 recognized species have been cytogenetically examined (Table 1). The diploid number among Gekkonid lizards ranges from 2*n*=16 to 2*n*=46 with most of the karyotypes composed of 28-46 chromosomes (Gorman 1973, Schmid *et al*. 1994). The typical karyotype consists of a gradual series of acrocentric chromosomes which there is no difference between macro and microchromosomes (Molavi *et al*. 2014).

The occurrence of techniques related to DNA are encouraging for the advance of the comprehension of the animal genome structure and evolution (Martins *et al*. 2011; Barreto *et al*. 2021). Microsatellites are repetitive (in *tandem*) DNA sequences of one to six nucleotides found in all eukaryotic organisms (Cioffi and Bertollo 2012; López-Flores and Garrido Ramos 2012). In several species, these sequences can be found in long repetitions associated with heterochromatic regions (Martins 2007; Cioffi *et al*. 2011). This information on chromosomes is considered important along with other information for the identification of the species (Campiranont 2003). The cytogenetic analysis of the higher molecular chromosome structure can provide invaluable insight for the management of threatened species, where DNA alone could not address all genetic risks and threats to populations (Potter and Deakin 2018). The distribution of microsatellites on chromosomes could help on the elucidation of evolutionary processes that lead to a karyotypic macrostructure differentiation and even to the origin of sex chromosomes systems (Cioffi *et al*. 2011), where the investigation of the sex-determining system was finished only one species, the Bornean endemic *Cyrtodactylus pubisulcus* through traditional cytogenetics (Ota *et al*. 1992; Keating *et al*. 2021)

Accordingly, the present study is the first cytogenetic study on *C. inthanon* from Thailand accomplished with classical and molecular cytogenetic techniques.

Table 1. Karyotype reviews in the genus *Cyrtodactylus*.

Species	2*n*	NF	Karyotype	NOR	Locality	Reference
C. consobrinus	48	50	2bi-arm+46t	-	Malaysia	Ota *et al*. (1992)
C. doisuthep	34	56	14m+6sm+2a+12t	P9, 13	Thailand	Thongnetr *et al*. (2021)
C. interdigitalis	42	52	4m+2sm+4a+32t	P12	Thailand	Thongnetr *et al*. (2019a)
C. inthanon	**40**	**58**	**12m+4sm+2a+22t**	**P12**	**Thailand**	**Present study**
C. jarujini	40	56	8m+4sm+4a+24t	P13, 14	Thailand	Thongnetr *et al*. (2021)
C. kunyai	40	52	8m+4sm+6a+22t	P12	Thailand	Thongnetr *et al*. (2019a)
C. pubisulcus	42	44	2bi-arm+40t	-	Malaysia	Ota *et al*. (1992)
C. saiyok	42	42	42t	P15	Thailand	Thongnetr *et al*. (2019b)

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

Data provided here will increase our knowledge of cytogenetic information which can be used as a basis to comprehensively examine the taxonomy and evolutionary relationship of *Cyrtodactylus* species and other gekkonids.

MATERIALS AND METHODS

Sample collection and chromosome preparation

Five male and five female specimens of *C. inthanon* were collected from the Doi Inthanon reinforces, Chiang Mai Province, northern Thailand (Fig. 1). All bent-toed geckos were transferred to the laboratory and kept under standard conditions for one day prior to the experimentation.

Chromosomes were directly prepared *in vivo* (Ota *et al*. 1990) by 0.1% colchicine were injected into the geckos' intramuscular and abdominal cavity and then left for 8-10 hours. Bone marrow (in male and female) and testis (male) 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.

Giemsa's staining, Ag-NOR banding technique and Chromosome analysis

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). Then, the slides were rinsed thoroughly with running tap water to remove excess stain.

Two drops of each 50% silver nitrate and 2% gelatin were droped on slides, respectively. Then it was sealed with cover glasses and incubated at 60 °C for 5-10 minute. There after that it was soaked in distilled water until cover glasses are separated. (Howell and Black 1980).

Ten clearly observable metaphase cells with well spread chromosomes of each male and female were selected and photographed. The length of short arm chromosome (Ls) and 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 standard deviation (SD) of RL and CI were analysed 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 karyotyping and idiograming.

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: $d(CA)_{15}$, $d(GC)_{15}$, $d(CAG)_{10}$ and $d(GAA)_{10}$ were directly labelled 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 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 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

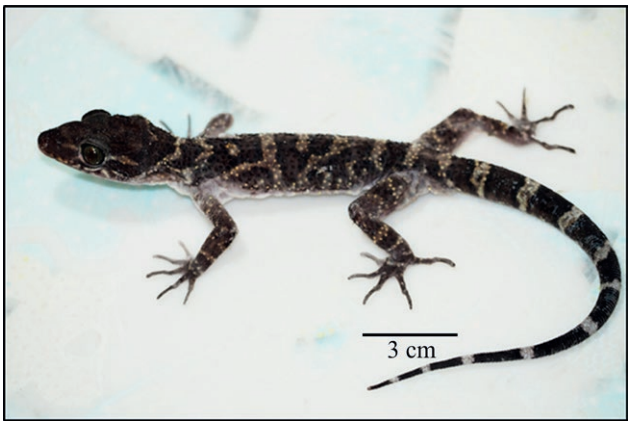

Figure 1. General characteristic shape of the *Cyrtodactylus Inthanon*, the most prominent feature in the top view.

with DAPI (1.2 μg/mL), mounted in antifading solution (Vector, Burlingame, CA, USA) and analyzed in fluorescence microscope Nikon ECLIPSE.

RESULTS

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

The diploid number and NF in *C. inthanon* are 40 and 58, respectively. The karyotype of *C. inthanon* is composed of 12 metacentrics, 4 submetacentrics, 2 acrocentrics and 22 telocentrics There are exhibited no sex differences in karyotypes between males and females. (Table 2 and Fig. 2A-D).

Nucleolar organizer region and meiotic cell characteristics

The determination of chromosome marker for this species is firstly obtained by using the Ag-NOR banding technique. The nucleolar organizer regions (NORs) are observed on the telomeric region of the acrocentric chromosome pair 12 both male and female (Fig. 2 E-H). The meiotic cell of *C. inthanon* reveals the diplotene phase (Fig. 3), which shows synapsis between two the homologous and compacted chromosomes. The metaphase I (meiosis I, reductional division) can be defined as the 20 bivalents

Patterns of microsatellite

The microsatellite $d(CA)_{15}$ presents the signals highly accumulated at the interstitial subcentromeric region on short arms of metacentric chromosome pair 10 (Fig. 4A) whereas, the microsatellite $d(GC)_{15}$, $d(CAG)_{10}$ and $d(GAA)_{10}$ are distributed weak signals throughout the whole chromosomes (Fig. 4C-D).

DISCUSSION

From the previous reports, the chromosome exhibited various number in the *Cyrtodactylus*, ranging from 34 to 48, however, the most frequent numbers were 40 and 42. The present study showed that the chromosome number of *C. inthanon* were 40. This result revealed accordance with other species that have been reported, such as *C. jarujini* and *C. kunyai* (Thongnetr *et al*. 2019a, 2021). Moreover, the diploid chromosome number (2*n*) differed from *C.*

Table 2. Mean length of short arm chromosome (Ls), length of long arm chromosome (Ll), length of total chromosomes (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) from 10 metaphases of male and female *Cyrtodactylus inthanon*, 2*n*=40.

Chr. pair	Ls	Ll	LT	CI±SD	RL±SD	Chr. size	Chr. type
1	3.568	4.695	8.263	0.567±0.026	0.101±0.008	Large	metacentric
2	2.325	4.177	6.503	0.640±0.019	0.079±0.003	Large	submetacentric
3	2.059	3.799	5.858	0.648±0.023	0.072±0.002	Large	submetacentric
4	0.000	5.921	5.921	1.000±0.000	0.072±0.005	Large	telocentric
5	0.000	5.894	5.894	1.000±0.000	0.072±0.003	Large	telocentric
6	0.000	5.330	5.330	1.000±0.000	0.065±0.004	Large	telocentric
7	0.000	4.827	4.827	1.000±0.000	0.059±0.003	Medium	telocentric
8	0.000	4.302	4.302	1.000±0.000	0.052±0.002	Medium	telocentric
9	0.000	3.994	3.994	1.000±0.000	0.049±0.002	Medium	telocentric
10	1.688	2.476	4.164	0.595±0.021	0.051±0.002	Medium	metacentric
11	0.000	3.786	3.786	1.000±0.000	0.046±0.002	Medium	telocentric
12*	1.069	2.431	3.500	0.705±0.070	0.043±0.003	Small	acrocentric
13	0.000	2.785	2.785	1.000±0.000	0.034±0.003	Small	telocentric
14	0.000	2.707	2.707	1.000±0.000	0.033±0.001	Small	telocentric
15	0.000	2.334	2.334	1.000±0.000	0.028±0.004	Small	telocentric
16	1.202	1.698	2.900	0.584±0.035	0.036±0.004	Small	metacentric
17	0.000	2.166	2.166	1.000±0.000	0.027±0.003	Small	telocentric
18	1.087	1.512	2.599	0.582±0.025	0.032±0.003	Small	metacentric
19	0.916	1.284	2.200	0.586±0.016	0.027±0.003	Small	metacentric
20	0.796	0.999	1.795	0.557±0.075	0.022±0.001	Small	metacentric

Remark: Chr. = chromosome; * = satellite chromosome (nucleolar organizer region, NOR).

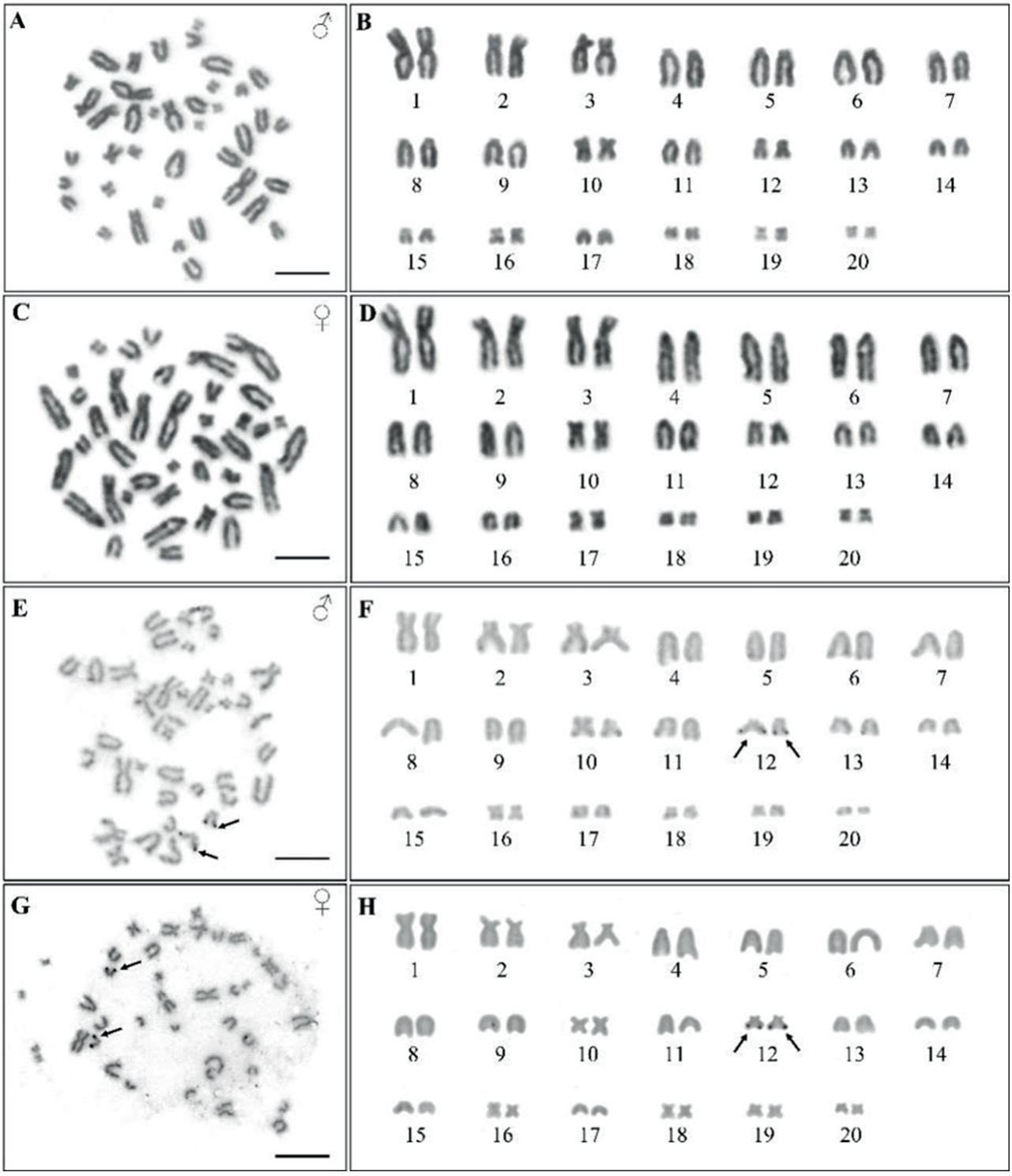

Figure 2. Metaphase chromosome plates and karyotypes of male and female *Cyrtodactylus inthanon,* 2*n*=40 by conventional staining (A-D) and Ag-NOR banding technique (E-H). Scale bars indicate 5 micrometers. The arrows indicate nucleolar organizer regions/NOR.

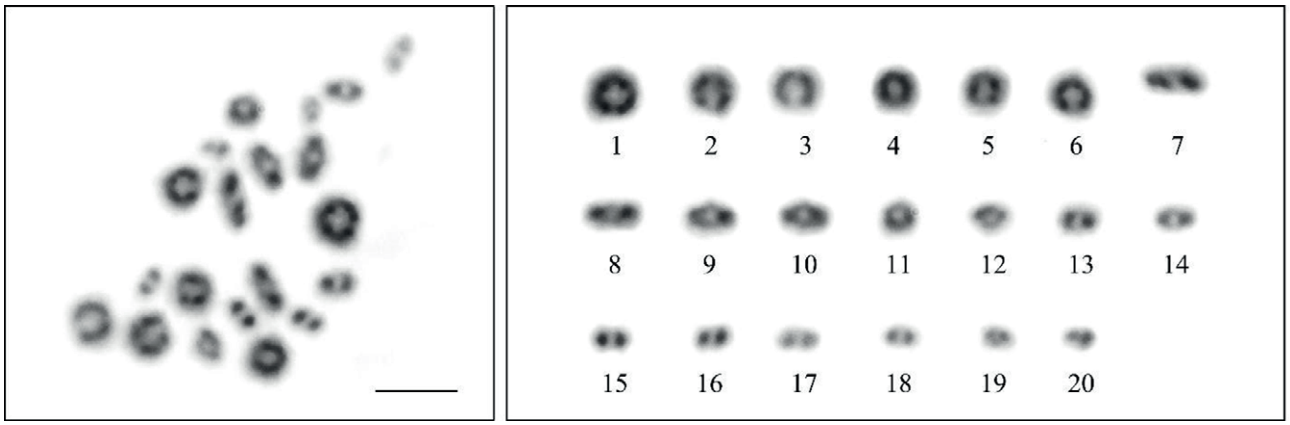

Figure 3. Metaphase I chromosome plate and karyotypes of *Cyrtodactylus inthanon*, *n*=20 by conventional staining technique. Scale bars indicate 5 micrometers.

doisuthep (2*n*=34), *C. interdigitalis*, *C. pubisulcus, C. saiyok* (2*n*=42) and *C. consobrinus* (2*n*= 48) (Ota *et al*. 1992, Thongnetr *et al*. 2019a, 2019b, 2021). The different *Cyrtodactylus* species underwent an extremely diversified karyotype evolution, considering the numerical and structural aspects of their complements, with the NF that varied from 42 to 58. The karyotype of *C. inthanon* is composed of 12 metacentric, 4 submetacentric, 2 acrocentric and 22 telocentric chromosomes. The karyological characteristics of *C. inthanon* obtained in the present study is the first report of chromosome sizes and the chromosome types. In other species of *Cyrtodactylus*, the different karyological structure can be found as well. However, the karyotype of *C. inthanon* resemble other *Cyrtodactylus* and other gekkonids, which comprised many gradient mono-armed (telocentric) and few bi-armed chromosomes (meta- or submetacentric). The proximity of chromosome number and karyotype feature within genus *Cyrtodactylus* represents a close evolutionary line in the group. (Trifonov *et al*. 2011). This analysis was performed to highlight the combined importance of the different chromosome rearrangements in the evolutionary modeling of their karyotypes, such as Robertsonian rearrangements or centric fission (Ueno and Takai 2000) fusion and especially, pericentric inversions (Jacobina *et al*. 2011).

The nucleolar organizer regions (NORs) represent the location of genes that have a function in ribosome synthesis (18S and 28S ribosomal RNA). NORs of *C. inthanon* exhibited a single pair of Ag-NORs located on the telomeric region on the long arm of the acrocentric and are similar to the previous reports of the genus *Cyrtodactylus,* e.g., *C. interdigitalis, C. kunyai* (Thongnetr *et al*. 2019a, 2021).

The metaphase I (meiosis I, reductional division) was found in *C. inthanon*, which showed as the 20 bivalents (Fig. 3). No metaphase I cell with partially paired bivalents, which are speculated to be male heteromorphic sex chromosomes in this species. From this result, the behavior and number of chromosomes in metaphase I confirmed of each other's accuracy and also verified the accuracy of diploid chromosomes in somatic cells.

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 showed abundant distribution throughout eukaryotic genomes, being dispersed or clustered both in euchromatin or heterochromatin. They are highly polymorphic regarding copy number variations (Ellegren 2004). In our present study in *C. inthanon* exhibited the microsatellite d(CA)15 was revealed that the signals highly accumulated at the interstitial subcentromeric region on short arms of metacentric chromosome pair 10 (Fig. 4A), whereas, the microsatellite d(GC)15, d(CAG)10 and d(GAA)10 were distributed weak signals throughout the whole chromosomes (Fig. 4C-D). In previous study, microsatellite pattern (the dinucleotides $d(A)_{20}$, $d(CAG)_{10}$, $d(CGG)_{10}$, $d(GAA)_{10}$ and $d(TA)_{15}$) on *C. jarujini* and *C. doisuthep* accumulated exclusively in telomeric and subtelomeric chromosomal regions bearing dispersed over the whole genomes including chromosomes and some had strong signals on only a pair of homologous chromosomes (Thongnetr *et al*. 2021). However, the results clearly indicate 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).

The first cytogenetic study of the *C. inthanon* has enabled us to delineate the process of chromosomal reorganization in this group chromosome system and

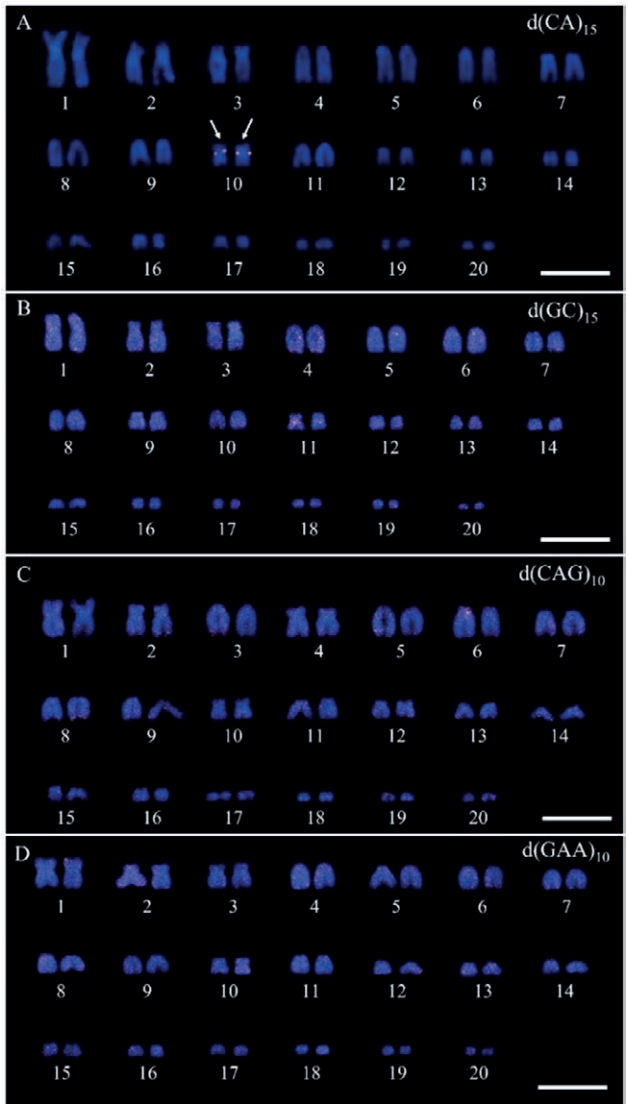

Figure 4. Karyotypes of *Cyrtodactylus inthanon*, 2*n*=40 presenting the patterns of microsatellite $d(CA)_{15}$, $d(CAG)_{10}$, $d(GAA)_{10}$ and $d(GC)_{15}$ (A-D). Scale bars: 5 μm.

FISH mapping. The results obtained here can be used to support the further investigation on taxonomy, biodiversity conservation and evolutionary relationship among the genus *Cyrtodactylus* and others.

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