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Chromosome Aberrations in the Karyotype of the Southeast Asian Mangrove Frog *Fejervarya cancrivora* (Gravenhorst, 1829) from Luzon Island, Philippines

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Abstract: ***Chromosome analysis of 5, 100 bone marrow cells of F. cancrivora revealed that this species possessed an asymmetrical karyotype with diploid number 2n = 26 typical of frogs belonging to the Genus Rana. Nombre fundamental (NF) was 52, consisting of 26 homomorphic chromosomes. Karyotype consisted of three (3) pairs of metacentric and ten (10) pairs of sub-metacentric chromosomes. Sex chromosomes were not morphologically distinct with regard to size and structure. Testis tissue preparations from male specimens showed 26 chromosomes in spermatogonia. Primary spermatocyte at meiotic metaphase – I (at diakinesis), and secondary spermatocyte at meiotic metaphase - II consisted of 13 ring- and 13 haploid chromosome pairs. Nucleolar Organizer Region (NOR) was detected at the q-arm of chromosome pair 9. The karyotype formula of F. cancrivora was determined to be: 2n (26) =*** L_5^{sm} + S_2^{sm} + S_1^{m} + S_2^{sm} + S_1^{m} + S_1^{sm} + S_1^{m}. ***Chromosome aberrations were scored for 0.4% of the cells analyzed. The most frequent aberration was the gap, followed by the break and minutes. Gonadal cells did not manifest any aberrations in their chromosomes. F. cancrivora could become a biological monitor to evaluate pollution and environmental degradation similar to recent work undertaken on Fejervarya limnocharis.***

Keywords: Biological monitor, Chromosome, *Fejervarya cancrivora*, Karyotype

1. Introduction

The Southeast Asian mangrove frog, *F.* (*Rana*) *cancrivora* (Gravenhorst, 1829) is widely distributed throughout Asia, extending from the countries of Bangladesh (Kurniawan et al., 2011), India (Satheeshkumar, 2011), Thailand, coastal Southern China, Philippines, Indonesia (Kurnianti and Sulistyadi, 2017) and Malaysia. It was introduced into Taiwan (inaturalist, 2000) and Guam (Christy et al., 2007). Its widespread distribution is due to its ability to tolerate temperatures of up to 42 ^{o}C (Dabruzzi *et al*, 2012) and thrive in saline aquatic habitats (Schmidt-Nielsen and Lee, 1962). This euryhaline frog species thrives in mangrove swamps and fish ponds, often competing for food and space with *F. limnocharis* in rice fields.

Economically speaking, *F. cancrivora* is the major species (75%) shipped by Indonesia to Asian and European countries, with annual production at 5, 600 tons in 1992. Frog leg exports originated from wild harvests of *F. cancrivora*, followed by less abundant species such as *F. limnocharis* and *Limnonectes macrodon* (Kusrini and Alford, 2006).

F. cancrivora is a stocky bodied frog with brownish olive color, with whitish to yellowish under parts and dark mottlings. It possesses a small, elongated skin flap at the edge of the distal tarsal (Yodthong et al., 2019) that is not present in *F. limnocharis*. Males have paired, black triangular pigmentation at the ventral edge of the mouth.

Average length and weight of individuals used in this study varied between 12.5 to 61.5g, with females being larger than males. In Indonesia wild females are larger than males: snout vertical length (mm) and weight (g) ranges between: 75.1mm, 48.5g in females *versus* 65.54mm, 29.5g in males (Kusrini and Alford, 2006).

Females were more prevalent than males in wild *F. cancrivora* captured for this study. Wischi (1929) speculated that elevated temperatures caused several *Rana sylvatica* female tadpoles to develop into hermaphrodites, then the rest developed into males, resulting in more females in wild populations (Eggert, 2004).

The wide distribution of *F. cancrivora* throughout Asia (Kurniawan *et al.*, 2011) has resulted in the development of three genotypes due to evolution in different climactic regimes throughout time. Nonetheless slight changes in the external skin color and pigmentation of *F. cancrivora* have been observed throughout the geographic regions of distribution.

Cytogenetic studies on effects of geographical regimes to *F. cancrivora* chromosomes are limited as of this time of writing. Chromosome analysis is a cost-effective method to determine the effects to frogs of harmful substances released into the environment. Frog chromosomes are among the largest chromosomes in the vertebrate kingdom with lengths of 10um. Karyotypes of *Ranaspp.* usually consist of only thirteen pairs enabling researchers to analyze chromosome aberrations.

2. Materials and Methods

Collection and Transport of Live *F. cancrivora*

Seventy (70) *F. cancrivora* were purchased from frog collectors in three (3) fish pond sites in river tributaries of

Manila Bay, Luzon Island, Philippines: [1] San Pascual, Hagonoy, Bulacan, [2] Santa Ana, Bulacan, Bulacan, and [3] Libid, Binangonan, Rizal. Frogs were transported to the Laboratory in net bags.

Colchicine Administration into Frogs
A 0.1% colchicine solution administered at 1mL / 20g body weight (Mindrescu and Giohiorghita, 2008) was injected intra-peritoneally at the ventral side above the abdominal muscles. After two (2) hours, femur of males and females, and testis of males were removed, then transferred to 15mL centrifuge tubes.

Hypotonic Treatment, Centrifugation and Fixation of Cells
Ten (10) mL of 0.075M KCl solution (Patawang *et al.*, 2014) was added to the 15mL centrifuge tubes containing bone marrow. Bone marrow was flushed from the femur using a fine needle syringe and teased using a glass rod until the solution turned milky. Test tubes were let alone for 30 minutes then centrifuged at 1, 000 rpm for 10 minutes (Patawang *et al.*, 2013). Supernatant was discarded, leaving the cell pellet at the bottom of the centrifuge tube. The tube was vortexed to disturb the pellet. Gradually, 10mL fixative (3:1 ethanol glacial acetic acid + 1 drop chloroform per 1ml ethanol) was added into the test tube while constantly shaking the pellet. The test tube was centrifuged at 1, 000 rpm for 10 minutes, first-fixative solution removed and replaced with 10mL new fixative. Fixative replacement was repeated at least three times until the cell pellet was fully dehydrated, leaving one (mL) fixative - cell suspension. Consequently, the fixative for testis tissue preparations did not contain chloroform.

Chromosome Plate Preparation, Drying and Staining
After hypotonic treatment and fixation, bone marrow and testis cell suspensions were dropped six inches above pre-cleaned and warmed 25 x 76mm slides (to splash chromosomes in fixed cells), dried in a slide drier, stained with 0.1% toluidine blue solution. A 24 x 56mm cover slip was placed over the stained slide to enable microscopic analysis under100x oil immersion objective.

Karyotyping
One hundred (100) bone marrow cells in metaphase-C per frog were analyzed under a microscope for chromosome numbers, shapes and sizes. Chromosome pairs were arranged in descending size and according to the position of the centromere.

The karyotype was derived from good chromosome spreads from each of the 51 frog specimens. Modal number (M) (number of cells having the highest chromosome frequency count) was determined.

Karyotypes from testis chromosomes (*i.e.,* spermatogonia, primary and secondary spermatocytes) were also produced and compared to chromosomes from bone marrow preparations.

Statistical Design

Chromosome Arm Measurements
Lengths of the short (p-) and long (q-) chromosome arms of bone marrow cells were measured to obtain mean values for Average Length (ĀL), Relative Length (ŘL), Arm Ratio (ĀR) and Centromeric Index (ĈI), where:

$$AL_{\text{chromosome n}} = \frac{\Sigma (p+q)_{\text{chromosome n [cell no. 1]}} + (p+q)_{\text{chromosome n [cell no. 2]}} + \ldots + (p+q)_{\text{chromosome n [cell no. 51]}}}{51}$$

$$RL_{\text{chromosome n}} = \frac{p+q \times 100}{\text{Length of haploid set}}$$

$$AR_{\text{chromosome n}} = \frac{q_{\text{chromosome n}}}{p_{\text{chromosome n}}}$$

$$CI_{\text{chromosome n}} = \frac{p_{\text{chromosome n.}}}{p_{\text{chromosome n}} + q_{\text{chromosome n}}}$$

Nombre Fundamental (NF) = number of major chromosome arms of the 2n set.

Chromosome Aberration Count
Frequency counts for bone marrow and testis cells with chromosome aberrations on the q- and p- arms were done based on aberration type.

3. Results and Discussion

Modal Diploid Chromosome Number
F. cancrivora caught in three remote sites in Luzon produced a modal diploid chromosome number 2n = 26. (Table 1). Fifty-one (51) (14 male, 37 female) out of 70 sacrificed frogs yielded good chromosome preparations. Of the 5, 100 metaphase spreads from bone marrow preparations, 4, 183 (82%) had the characteristic count of 26; 461 (9%) possessed 25 chromosomes; 271 (5%) contained 24 chromosomes; 125 (2%) had 23 chromosomes; 52 (1%) showed 22 chromosomes; 8 (0.2%) had 27 chromosomes. The modal diploid chromosome number was 2n = 26.

***F. cancrivora* Karyotype**
Bone marrow
The karyotype of *F. cancrivora* comprised 5 pairs of large chromosomes and 8 pairs of small chromosomes. The largest pair measured 10.39µm while the smallest was 2.31µm divisible into 4 groups. Group A comprised the first 5 large chromosome pairs, group B consisted of pairs 6 – 8, group C contained pairs 9 – 11 while group D was composed of the smallest pairs 12 to 13.

Fifty-one (51) bone marrow C-metaphase plates showed 13 pairs of homomorphic chromosomes. Karyotype consisted of three (3) pairs of metacentric and ten (10) pairs of sub-metacentric chromosomes (Figure 1 [A]).

Table 1: Chromosome counts of *F. cancrivora* bone marrow preparations. Column with dark border indicates modal diploid chromosome number was 2n = 26

Source	Cells Examined			2n number of Chromosomes						
	Male	Female	Subtotals	22	23	24	25	26	27	28
Binangonan, Rizal	400	2, 300	2, 700	21	58	128	249	2, 239	5	0
Bulacan, Bulacan	600	400	1, 000	5	26	62	109	797	1	0
Hagonoy, Bulacan	400	1, 000	1, 400	26	41	81	103	1, 147	2	0
Totals	1, 400	3, 700	5, 100	52	125	271	461	4, 183	8	0

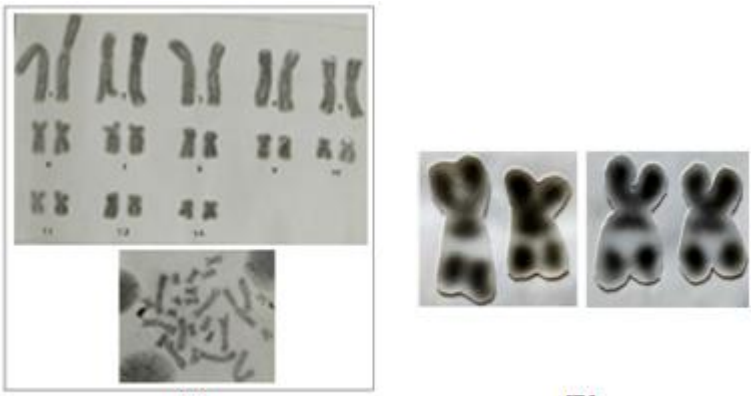

Figure 1: [A] Female *F. cancrivora* bone marrow chromosomes. [B] NOR chromosome pair 9 of male (left) and female (right), respectively

Table 2: Mean values for p-arm, q- arm, Average Lengths (ĀL), Relative Lengths (ŘL), Arm Ratio (ĀR), Centromeric Index (ĈI) and shape of *F. cancrivora* bone marrow chromosomes

Chromosome pair no. (n)	Group	Mean (µm)		ĀL (µm)	ŘL	ĀR	ĈI x 100%	Chromosome Shape
		p-	q-	ĀL ± S.D.	ŘL ± S.D.	ĀR ± S.D.	ĈI ± S.D.	
1	A	4.57	5.82	10.39 ± 0.84	15.41 ± 0.41	1.28 ± 0.03	44.0 ± 1.0	Sub-metacentric
2		3.53	5.29	8.82 ± 0.71	13.09 ± 0.28	1.51 ± 0.04	40.0 ± 1.0	Sub-metacentric
3		3.15	4.73	7.88 ± 0.63	11.70 ± 0.25	1.51 ± 0.05	40.0 ± 1.0	Sub-metacentric
4		2.15	5.26	7.41 ± 0.58	11.01 ± 0.30	2.46 ± 0.15	29.0 ± 1.0	Sub-metacentric
5		2.95	4.07	7.02 ± 0.51	10.43 ± 0.14	1.40 ± 0.04	42.0 ± 1.0	Sub-metacentric
6	B	1.59	2.49	4.08 ± 0.31	6.06 ± 0.19	1.59 ± 0.08	39.0 ± 1.0	Sub-metacentric
7		1.13	2.65	3.78 ± 0.28	5.62 ± 0.15	2.37 ± 0.16	30.0 ± 1.0	Sub-metacentric
8		1.69	1.99	3.68 ± 0.26	5.47 ± 0.14	1.20 ± 0.09	46.0 ± 2.0	**Metacentric**
9	C	1.34	1.92	3.26 ± 0.26	4.87 ± 0.21	1.48 ± 0.07	41.0 ± 1.0	Sub-metacentric
10		1.03	2.01	3.04 ± 0.24	4.53 ± 0.16	1.93 ± 0.14	34.0 ± 2.0	Sub-metacentric
11		1.39	1.51	2.90 ± 0.22	4.32 ± 0.15	1.08 ± 0.05	48.0 ± 1.0	**Metacentric**
12	D	1.02	1.73	2.75 ± 0.21	4.10 ± 0.14	1.74 ± 0.10	37.0 ± 1.0	Sub-metacentric
13		1.09	1.22	2.31 ± 0.18	3.45 ± 0.14	1.12 ± 0.06	47.0 ± 1.0	**Metacentric**

Table 3: Morphological characteristics of *F. cancrivora* chromosomes

Group	Pair no. (n)	Characteristics
A	1	Largest of the chromosome; centromere located in the median region; sub-metacentric.
	2	Second largest chromosome; centromere located in the median region; sub-metacentric.
	3	Third largest chromosome; centromere located in the median region; sub-metacentric.
	4	Fourth largest chromosome; centromere located in the sub-median region; sub-metacentric.
	5	Fifth largest chromosome; centromere located in the median region; sub-metacentric.
B	6	Largest of the small chromosomes; centromere located in the median region; sub-metacentric.
	7	Seventh small chromosome; centromere located at the sub-median region; sub-metacentric.
	8	Eighth small chromosome; centromere located in the median region; metacentric.
	9	Ninth small chromosome; NOR chromosome; secondary constricted located on long arm; centromere located in the median region; sub-metacentric.
C	10	Tenth small chromosome; centromere located in the sub-median region; sub-metacentric.
	11	Eleventh small chromosome; centromere located in the median region; metacentric.
D	12	Twelfth small chromosome; centromere located in the sub-median region; sub-metacentric.
	13	Smallest chromosome; centromere located in the median region; metacentric

Sex Chromosomes

The basic staining technique could not determine the sex chromosomes of *F. cancrivora* by morphological features or measurements. Chromosomes of the male and female karyotypes (Figure 3) were homomorphic in the case of *F. cancrivora* and did not reveal any unique characteristics to distinguish the male from the female karyotype. Frogs exhibit either the XX / XY (*e.g. F. limnocharis*: Patawang et al., 2014) or the ZZ/ZW (*e.g. Bufomarinus*: Abramyan and Ezaz, 2009; *Pseudistocatin*: Gatto et al., 2016) system. Sex chromosomes of most frogs are homomorphic (Chang et al., 2017; Ganzoni et al., 2018), but karyotypes with heteromorphic sex chromosome pairs have been reported by several authors (Patawang et al., 2014; Skorinov et al., 2020). Regarding the heteromorphic sex chromosomes of *F. limnocharis* (2n = 26), the X chromosome was metacentric and slightly larger than the sub-metacentric Y chromosome (1.03 *vs.* 0.91µm, respectively).

C-banding of the largest pair of homomorphic chromosomes in *Proceratophrysbioei* revealed a ZZ/ZW system based on constitutive heterochromatin distribution. Females possessed a pair of ZZ chromosomes extensively covered with heterochromatin. On the other hand, heterochromatin concentration was located only at the centromeric region of the W chromosome (Ananias et al., 2007).

Arm Measurements

Table 2 summarizes the mean values for Average Length (ĀL), Relative Length (ŘL), Arm Ratio (ĀR) and Centromeric Index (ĈI). The idiogram of *F. cancrivora* consisted of 1 group large (A) and 3 groups small (B, C, D) chromosomes. *F. cancrivora* possessed a simple karyotype of metacentric and sub-metacentric chromosomes (Table 3).

Relative Length analysis indicated that 15.41% of the entire genome was located in chromosome pair no. 1. Chromosome pair no. 13 contained only 3.45% of the total haploid set. Arm Ratio and Centromeric Index showed that there were 3 metacentric pairs (all of which were small chromosomes) and 10 sub-metacentic pairs. *F. cancrivora* did not possess acrocentric or telocentric chromosomes. The values for Relative Lengths and Centromeric Indices revealed that there was little or no overlap of chromosome lengths in the karyotype of *F. cancrivora* (Figure 2).

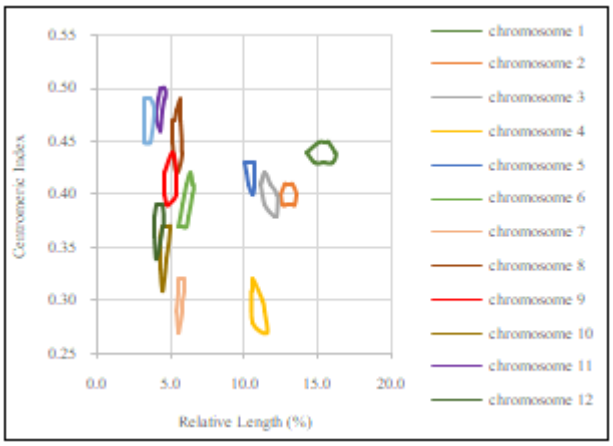

Figure 2: Relative Index and Centromeric Index data clusters of *F. cancrivora* do not show overlaps among chromosome pairs.

The *nombre fundamental* (NF) of *F. cancrivora* was 52, comprising of 6 metacentric and 20 sub-metacentric chromosome pairs.

The karyotype formula of *F. cancrivora* was therefore determined to be: 2n (26) = L_5^{sm} + S_2^{sm} + S_1^{m} + S_2^{sm} + S_1^{m} + S_1^{sm} + S_1^{m}. The idiogram of *F. cancrivora*is shown in Figure 3.

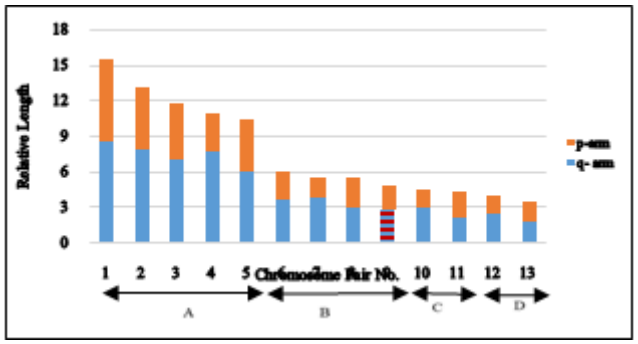

Figure 3: Idiogram of *F. cancrivora* chromosomes based on Relative Length. Orange stands for short arm and blue for long arm. Pair no. 9 with red-banded long arm is the NOR chromosome

NOR Chromosome Pair

Nucleolar organizer regions (NORs) in chromosomes are not stained and appear as gaps with dark satellite-like protruberances. NOR size differences are a constitutional, intra-individual characteristic that shows that a large number of amphibians are frequently heterozygous as far the number of rRNA genes on each half of the bivalent is concerned (Schmid, 1982). In closely-related anuran species, the NOR is located on the same chromosome pair (Schmid, 1983; Mahoney and Robinson, 1986).

The nucleolar organizer region (NOR) chromosome of *F. cancrivora* was pair no. 9 (Figure 1 [B]). The existence of only 1 pair of NOR chromosomes is a usual characteristic of primitive and highly evolved anurans (Schmid, 1982).

In both male and female *F. cancrivora* specimens the NOR chromosome pairs exhibited heteromorphisms in the size of the secondary constrictions similar to observations by Quindere *et al.*, (2009) on *Physalaemus cuvieri*. In *F. cancrivora* the NOR occupied an interstitial position at the long arms of chromosome pair no. 9. Consequently, the NOR of *Litoria nyakalensis* (2n=26) was also found at the long arms of chromosome pair no. 9 (Kakampuy *et al.*, 2013). In *F. limnocharis* (2=26) the NOR was located at the small arms of chromosome pair no. 6 (Patawang *et al.*, 2014). In contrast, the NORs of the hylid frog *Litoria genimaculata* (2n = 26) were located at the small arms of chromosome pair no. 7.

Testis Preparations

Sex chromosomes of *F. cancrivora* in diploid spermatogonial cells were not morphologically distinct. The karyotype was similar to those of bone marrow preparations.

Meiotic metaphase – I – at – diakinesis in most anuran species is characterized by chromosome bivalents that assume the shape of rings (Schmid *et al.*, 1989). Chromosomes of *F. cancrivora* primary spermatocytes were joined together at their terminal ends, comprising 5 large and 8 small rings. Quadrivalent ring chromosomes such as those found in the hylid frog *Aplastodiscus pervidis* (Gruber et al., 2012) were not detected. No morphologically – distinct allosome pair was observed in *F. cancrivora* primary and secondary spermatocytes (Figures 4 and 5).

Allozyme and molecular analysis of 16S rRNA identified 3 *F. cancrivora* types: a mangrove – type, a large – type, and a Pelabuhan Ratu/Sulwesi type (Kurniawan et al., 2011). Meiotic metaphase – I chromosomes of the 3 types (including the mangrove – type from Luzon Island, Philippines –analyzed in this study) possessed 13 ring bivalents. In contrast, cross breeding geographically remote *F. cancrivora* individuals (*e.g.* Selangor - female x Khulna-male) resulted in bivalent rings or rods in the progeny's chromosomes.

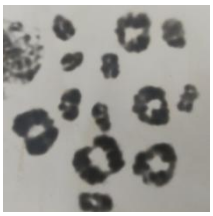

Figure 4: Primary spermatocyte of *F. cancrivora* at Meiotic Metaphase–I–at–diakinesis.

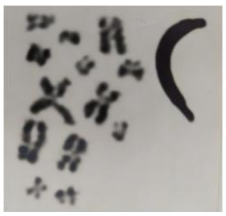

Figure 5: Secondary spermatocyte of *F. cancrivora* at meiotic Metaphase II.

Chromosome Aberration Analysis

This study observed chromosome aberrations in bone marrow preparations. Of the 5, 100 somatic metaphase plates analyzed, nineteen (0.37%) possessed gaps, breaks, or minutes (Table 4). Ninety-two (92) percent of the aberrations were located in large chromosomes belonging to Group A (Figure 6). The most common aberration found in *F. cancrivora* (this study) in *F. limnocharis* (Intamat *et.al.*, 2016; Boonmee et al., 2018) and in *H. rugulosus* (Tengjaroenkul et al., 2017) was the single chromatid gap. This was also the most frequent aberration scored by Gray et al. (1979) on tail cells of *Rana clamitans* tadpoles exposed to the mutagen Dispersion Yellow 3.

The aberrations scored for *F. cancrivora* resembled those kinds formed by exposing cells to chemical clastogens, viruses or ionizing radiation that may be present in the environment. Finding dicentric or ring aberrations would indicate damage to the genome in radiation dosimetry studies (Buckton and Evans, 1973). However, chromosome aberrations were not observed in cells undergoing meiotic metaphase I or metaphase II, when the diploid chromosome number was reduced to a haploid number n = 13. The bone marrow preparations of the wild *F. cancrivora* specimens did not show dicentric or ring aberrations induced by prolonged exposure to potent mutagens.

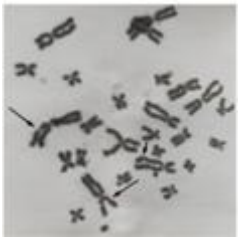

Figure 6: Iso-chromatid gap at the p- arms of a large chromosome (bottom arrow) and chromatid gaps at the q-arms of two large chromosomes (middle arrows).

Table 4: Chromosome aberrations observed in bone marrow metaphase plates of *F. cancrivora*. Italicized numbers represent aberrations found in small chromosomes

Aberration>	Minute	Chromatid Break		Iso-chromatid Break		Chromatid Gap		Iso-chromatid Gap	
Site, Weight, Sex		p	q	p	q	p	q	p	q
Binangonan, Rizal									
[01] 22.5g M							1		
[02] 12.5g M	2						1		
[03] 17.5g F						1			
[04] 30.0g F						1			
[05] 22.0g F							1		
[06]23.0g F						***1***	1		
[07] 21.0g F					1				
[08] 20.0g F							3		
[09] 34.6g F									
Bulacan, Bulacan									
[10] 16.7g M							1		
[11] 21.0g M							1		
[12] 15.5g M				***1***			1		
[13] 61.5g F									
[14] 40.5g F	1						2		
[15] 40.5g F					1				
[16] 22.5g F							1		
[17] 22.5g F									
Hagonoy, Bulacan									
								1	
[18] 17.5g M							1		1
[19] 14.5g F									
24 / 5, 100 Cells	3	0		3		17		2	

Studies on chromosome aberrations in *F. limnocharis* frogs exposed to arsenic contamination in gold mine wastes detected single iso-chromatid gaps, single chromatid breaks, deletion, fragmentation, centric fragmentation and polyploidy (Intamat et al., 2016). In rice fields contaminated with the organophosphate pesticide Chlorpyrifos, chromatid gaps, breaks, deletions, gapsand centric fragmentations were found in *Hoplobatrachus rugulosus* bone marrow chromosomes (Tengjaroenkul et al., 2017). *F. limnocharis* exposed to cadmium chloride developed single and iso-chromatid gaps, breaks, iso-arm fragmentation, chromatid decomposition, centric fragmentation, centromere gap, deletion and fragmentation (Boonmee et al., 2018). Chromosomes of *F. limnocharis* collected in municipal landfills had chromatid and iso-chromatid gaps, chromatid and iso-chromatid breaks, centric fragmentation, deletion,

fragmentation, translocation, centromere gap, iso-arm fragmentation and single chromatid decompose (Phoonaploy, 2016).

4. Conclusion

F. cancrivora could qualify as a biological monitor to evaluate environmental degradation since it has a stable karyotype comprising sufficiently large (2.39 – 10.31µm) and prominent (sub-metacentric and metacentric) chromosomes, in contrast to other frog species that possess telocentric chromosomes. It could also serve as an indicator of radiation effects since it exhibited chromosome aberrations seen in laboratory-irradiated animals. Pollution monitoring using chromosome aberration analysis could also be undertaken using frogs exposed to environmental chemicals.

5. Future Scope

Further studies on *F. cancrivora* cytogenetics could be undertaken to determine the following: [1] sensitivity of the frog to different doses of radiation and chemical mutagens; [2] development of chromosome aberrations induced by climactic changes (*e.g.* increases or drops in temperature); [3] the consequences of phenotypic and genotypic alterations to the karyotype and to its survival. Chromosome effects due to nutritional chemicals ingested in foods with genome-altering substances (*e.g.* mitogens in seeds such as naturally occurring phyto-hemagglutinins) could also be explored in the near future.

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